REMARKS

I. Formal Matters

A. Status of Claims

Claims 47-52 are pending in the application. Claims 37-46 are canceled in this amendment. Claims 37-52 are rejected.

B. Claim to Priority

The Examiner has not acknowledged Applicants' claim to priority or receipt of the certified copies of the priority documents.

All three priority documents are of record and are available in PAIR. Accordingly, in the next communication, the Examiner is requested, respectfully, to acknowledge Applicants' claim to priority and receipt of the certified copies of the priority documents.

II. Detailed Action

In the following comments, (E)-[5-[2-[1-phenyl-1-(3-pyridyl)methylideneaminoxy]ethyl]-7,8-dihydronaphthalen-1-yloxy]acetic acid and ONO-1301 are used interchangeably.

A. Claim Rejections - 35 U.S.C. § 112

1. Claims 37-52 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. According to the Examiner, the claims require that there be a nexus between the modulation of the VEGF and /or HGF receptor and a useful treatment of a disease/condition. The Examiner asserts, however, that the specification does not teach the necessary nexus.

Claims 37-46 have been canceled. Thus the rejection is moot as to these claims.

For the following reasons, the rejection is traversed as to method claims 47-52.

Initially, Applicants point out that claims 50-52 specifically recite that the compound is administered in an amount sufficient to accelerate production of VEGF and/or HGF. In this respect, it is known that acceleration of production of endogenous repair factors, such as VEGF and HGF are effective to treat various conditions, including acute pneumonia, pulmonary fibrosis, pulmonary hypertension, chronic obstructive pulmonary disease (COPD), and asthma, as recited in the claims. See specification, paragraph bridging pages 2-3.

In addition, the Examples in the specification demonstrate the activity of ONO-1301. Thus, Example 1 demonstrates that ONO-1301 accelerates hollow organ formation as efficiently as VEGF-A and HGF do. Example 2 demonstrates that ONO-1301 accelerates production of HGF protein and VEGF protein. Furthermore, Examples 4-7 demonstrate in vivo efficacy of ONO-1301 against ASO.

However, in order to further demonstrate that production of VEGF and HGF are effective to treat various conditions, including acute pneumonia, pulmonary fibrosis, pulmonary hypertension, chronic obstructive pulmonary disease (COPD), and asthma, Applicants provide the following discussion of the literature. A copy of each of the cited references is submitted herewith, pursuant to MPEP 609.05(c).

HGF (Hepatocyte Growth Factor) was discovered by Nakamura et al in 1984 (see *Biochemical and Biophysical Research Communications* (BBRC), 122, 1450 (1984)). Thereafter, several investigators proved that HGF has a very important role in tissue repair as a growth factor on alveolar blood capillaries and on alveolar epithelial cells after lung injury in the

lung as well as in the liver. Additionally, it has been shown that HGF acts on the structure of alveoli and peripheral tracheal epithelial cells in the fetal period (see *J Biochem*, 119, 591 (1996), *BBRC*, 241, 98 (1997) and *Development*, 125, 1315 (1998)). These results establish that HGF protects and cures bronchial epithelium cells from lung cell injury induced by acute or chronic inflammation and oxidative stress. Therefore, HGF is useful for the treatment of ALI (Acute Lung Injury), ARDS (Adult Respiratory Distress Syndrome), SIRS (Systemic Inflammatory Response Syndrome), pulmonary hypertension, COPD (Chronic Obstructive Pulmonary Disease) and asthma. On the other hand, it has been proven that HGF has a strong anti-fibrogenetic effect based on the inhibition of expression of TGF-β (transforming growth factor-β), which is important for the development of disease in chronic fibrous diseases (see *Nat Med*, 5, 226 (1999) and *J. Biol. Chem.*, 267, 24917 (1992)). Therefore, HGF has also been used for the treatment of pulmonary fibrosis and asthma as an anti-fibrogenetic agent of the alveolar and tracheal wall.

VEGF (Vascular Endothelial Growth factor) was discovered by Leung D. W. et al in 1989 (see *Science*, 246, 1306 (1989)). Thereafter, VEGF has been used for the treatment of peripheral artery disease (PAD) in gene therapy for vascularization (see *Lancet*, 348, 370 (1996)). Furthermore, VEGF has been used for wound healing in acute lung injury, tissue reproduction in COPD and vascularization in pulmonary hypertension.

The relationship between HGF/VEGF and each lung disease recited in the present claims is shown by numerous literature articles as summarized below.

Disease	HGF	VEGF
acute pneumonia	1) *J Biol Chem*, **268**, 21212 (1993) 2) *Am J Physiol*, **270**, 1031 (1996) 3) *Am J Respir Crit Care Med*, **162**, 707 (2000) 4) *Am J Pathol*, **155**, 949 (1999) 5) *Am J Respir Crit Care Med*, **158**, 386 (1998) 6)*Am J Physiol*, **278**, L382 (2000)	*7) Vascular endothelial growth factor (VEGF) is protective against acute lung injury ; Koh Hidefumi, Ishizaka Akitoshi, et al: ATS International Conference, Atlanta, Georgia, U.S.A ; 2002/05 8) *Eur Respir J*, **18**, 100 (2001)
pulmonary fibrosis	9) *Am J Respir Crit Care Med*, **156**, 1937 (1997) 10) *Respir Med*, **91**, 511 (1997) 11) *Respir Med*, **92**, 273 (1998) 12) *Am J Respir Cell Mol Biol*, **16**, 388 (1997)	13) *Am J Respir Crit Care Med*, **166**, 382 (2002.8)
pulmonary hypertension	14) *Circulation*, **106** (suppl I), I-264 (2002)	15) *Circulation*, **104**, 2242 (2001) 16) *Am J Respir Cell Mol Biol*, **23**, 762 (2000) 17) *FASEB J*, **15**, 427 (2001)
COPD	18) *Am J Respir Cell Mol Biol*, **26**, 525 (2002.5) 19) *Development*, **125**, 1315 (1998) 20) *Am J Respir Crit Care Med*, **160**, S72 (1999)	21) *Am J Respir Cri Care Med*, **163**, 737 (2001)
asthma	22) Japanese Journal of Allergology, **47**, 980 (1998) 23) *Am J Respir Crit Care Med*, **156**, 591 (1997)	

* 7) is a summary of a presentation at a scientific meeting, and a copy will be provided as soon as one is obtained.

Acute Pneumonia

The relationship between acute pneumonia and HGF is demonstrated in the following literature articles. *J Biol Chem*, 268, 21212 (1993), reports that HGF is newly produced in the lung after acute lung injury and may have a role in regeneration of the lung. *Am J Physiol*, 270, 1031L (1996), reports that HGF may act as a pulmotrophic factor responsible for airway and

alveolar regeneration during lung regeneration after acute lung injury. Furthermore, *Am J Respir Crit Care Med*, 162, 707 (2000) shows that HGF was induced in ischemic reperfused lungs and may play an important role in regeneration of injured lungs after pulmonary IR (ischemia-reperfusion). *Am J Pathol*, 155, 949 (1999) demonstrates that both HGF and KGF (keratinocyte growth factor) are present in the lung in greatly increased amounts soon after bleomycin-induced epithelial cell necrosis, and that these high levels are associated with both BR (bronchiolar epithelial) and alveolar epithelial cell proliferation. *Am J Respir Crit Care Med* 158, 386 (1998) shows that HGF and KGF are active in the alveolar space early in ALI (acute lung injury), probably mediating early events in lung repair, and that increased levels of HGF in edema fluid may have prognostic value early in ALI. *Am J Physiol*, 278, L382 (2000), indicates that fibroblast-derived HGF could be partially responsible for the changes in surfactant dysfunction seen in adult respiratory distress syndrome.

In ATS International Conference held in Atlanta, USA in 2002, Koh and Ishizaka et al. show VEGF is protective against acute lung injury. Furthermore, *Eur Respir* J, 18, 100 (2001) demonstrates that the initial phase of acute lung injury is associated with a decrease in vascular endothelial growth factor (VEGF) in the lung.

Pulmonary Fibrosis

The relationship between pulmonary fibrosis and HGF is shown in several literature publications. *Am J Respir Crit Care Med*, 156, 1937 (1997), suggests that HGF may be a potent candidate to prevent or treat lung fibrosis. Further, it has been shown that HGF may play an important role in the pathogenesis of idiopathic pulmonary fibrosis in *Respir Med*, 91, 511 (1997), *Respir Med*, 92, 273 (1998) and *Am J Respir Cell Mol Biol*, 16, 388 (1997).

Furthermore, *Am J Respir Crit Care Med*, 166, 382 (2002) reports that VEGF was significantly decreased in idiopathic pulmonary fibrosis, pulmonary fibrosis associated with a connective tissue disease, and sarcoidosis compared with non-smoking volunteers. The smoking patients showed a further decrease in VEGF. According to the authors, these data suggest that the decrease in VEGF in smokers and patients with chronic lung diseases may reduce angiogenesis and induce apoptosis of vascular endothelial cells.

Pulmonary Hypertension

In the relationship between pulmonary hypertension and HGF, it is suggested that therapeutic angiogenesis induced by HGF gene expression in the lung may be found suitable for treating subjects with decreased pulmonary vasculature or increased pulmonary vascular resistance in *Circulation*, 106 (suppl I), I-264 (2002).

As to the relationship between pulmonary hypertension and VEGF, it has been indicated that cell-based VEGF gene transfer is an effective method of preventing the development and progression of pulmonary hypertension in the monocrotaline model and this suggests a potential therapeutic role for angiogenic factors in the therapy of this devastating disease, *Circulation*, 104, 2242 (2001). Furthermore, *Am J Respir Cell Mol Biol*, 23, 762 (2000) concludes that adenoviral-mediated VEGF overexpression in the lungs attenuates development of hypoxic PH (pulmonary hypertension), in part by protecting endothelium-dependent function. In *FASEB J*, 15, 427 (2001), it is concluded that inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension.

COPD

With respect to the relationship between COPD(Chronic Obstructive Pulmonary Disease) and HGF, it is strongly suggested that HGF has a role as a pulmotrophic factor in postpneumonectomy compensatory lung regeneration, *Am J Respir Cell Mol Biol*, 26, 525 (2002). Furthermore, *Development*, 125, 1315 (1998), suggests that HGF is a putative candidate for a mesenchyme-derived morphogen regulating lung organogenesis and the authors found that HGF is involved in epithelial branching. Additionally, *Am J Respir Crit Care Med*, 160, S72 (1999) proposes novel approaches and targets for treatment of COPD, in which it is stated that HGF has a major effect on the growth of alveoli in fetal lung and it is possible that in the future, drugs might be developed that switch on responsiveness to HGF in adult lung or mimic the action of HGF.

As to the relationship between COPD and VEGF, it has been reported that VEGF, VEGF R2 protein, and mRNA expression was significantly reduced in emphysema. (applicant notes that "emphysema" is a major component of COPD.) *Am J Respir Cri Care Med*, 163, 737 (2001).

Asthma

Finally, as to the relationship between asthma and HGF, it has been reported that the HGF level in BALF (bronchoalveolar lavage fluid) in patients with asthma is high in contrast to the levels in patients in a control group *Japanese Journal of Allergology*, 47, 980 (1998). (An English translation of the publication is submitted herewith.) Furthermore, in *Am J Respir Crit Care Med*, 156, 591 (1997), it is suggested that TGF-β, but not EGF or GM-CSF, is involved in airways remodeling in asthma and chronic bronchitis.

From the evidence described above, it can be seen that a relationship between each lung disease recited in the claims and HGF/VEGF was known. The present inventors found for the

first time that ONO-1301 accelerates production of HGF and VEGF. Therefore, the present invention was accomplished based on the finding that ONO-1301 is useful for lung diseases relating to HGF and VEGF.

Accordingly, the Examiner is requested, respectfully, to reconsider and remove this rejection.

2. Claims 37-52 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. According to the Examiner, the art is so unpredictable, that in the absence of clinical data, it would require undue experimentation to determine whether (E)-[5-[2-[l -phenyl-1-(3-pyridyl)methylideneaminoxy]ethyl]-7,8-dihydronaphthalen-l-yloxy]acetic acid is useful to treat all of acute pneumonia, pulmonary fibrosis, pulmonary hypertension, chronic obstructive pulmonary disease (COPT), and asthma.

This rejection is moot as to canceled claims 37-46.

As to method claims 47 -52, the rejection is traversed, respectfully, for the same reasons discussed above. That is, production of VEGF and HGF is known to be effective to treat various conditions, including acute pneumonia, pulmonary fibrosis, pulmonary hypertension, chronic obstructive pulmonary disease (COPT), and asthma.

Accordingly, the Examiner is requested, respectfully, to reconsider and remove this rejection.

3. Claims 37-52 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. According to the Examiner, the instant specification does not provide a full description as to how much of a dose of the instant

compound is needed to treat the conditions recited in the claims or what dose is sufficient to accelerate production of VEGF and/or HGF.

For the following reasons, the rejection is traversed, respectfully.

The following description of dosages is found on page 32, lines 4-13 of the specification:

> Its dose varies depending on the age, body weight, symptoms, therapeutic effect, administration method, treating period and the like, but is usually within the range of from 1 ng to 100 mg per adult [], from once to several times a day by oral administration, or within the range of from 0.1 ng to 50 mg per adult [], from once to several times a day, from once to several times a week, or from once to several times in 3 months by parenteral administration in the form of a persistent preparation, or continuously administered into a vein within the range of from 1 hour to 24 hours a day. Since the dose varies under various conditions as a matter of course as described above, there is a case in which a smaller dose than the above range is sufficient or a case which requires the administration exceeding the range.

All diseases relating to the present invention can be treated by dosages within the ranges described above. Furthermore, it is common knowledge in the pharmaceutical arts that appropriate doses can be determined by routine experimentation, under each of the various conditions described above. The dose for administration for humans can be determined finally in clinical tests. Specifically, from Example 2 (Measurement of endogenous repair factor (HGF, VEGF) protein producing action (in vitro)) described on page 49 of the specification, it is apparent that ONO-1301 accelerates production of HGF and VEGF protein significantly in

concentrations of 10-7 mol/L. Furthermore, from Example 4 (Angiogenesis test (in vivo test) using rat leg ischemia (arteriosclerosis obliterans (ASO)) model) described on page 53 of the specification, it is apparent that ONO-1301 is effective by intramuscular injection of 1 mg/rat (ca. 3mg/kg). From these results, a person of ordinary skill in the art can estimate to some extent what would be an effective dose for humans.

Accordingly, the Examiner is requested, respectfully, to reconsider and remove this rejection.

B. Claim Rejections - 35 U.S.C. § 102

Claims 37-46 are rejected under 35 U.S.C. § 102(b) as being anticipated by Hayashi and JP 2000-239188 and JP 2000-86517. The Examiner states that all three of these documents disclose the instantly claimed compound in a composition for biological activity/study.

The rejection is moot as to canceled composition claims 37-46.

Claims 47-52 are method of treatment claims. Therefore the issue at hand is whether any of the cited references expressly or inherently discloses the claimed methods. As the Examiner knows, inherent anticipation requires that the reference necessarily teach the inherent feature. Thus, the reference would have to teach administration of (E)-[5-[2-[1 -phenyl-1-(3-pyridyl)methylideneaminoxy]ethyl]-7,8-dihydronaphthalen-l-yloxy]acetic acid to a subject that necessarily suffers from any of acute pneumonia, pulmonary fibrosis, pulmonary hypertension, chronic obstructive pulmonary disease (COPT), or asthma in a dose sufficient to treat any of these conditions, or with respect to claims 50-52, in a dose effective to accelerate production of VEGF and/or HGF.

For the following reasons, none of the cited references describes, expressly or inherently, the presently claimed methods.

1. Hayashi

Hayashi teaches that in crescentic-type anti-glomerular basement membrane nephritis in rats, antinephritic effects are observed on oral administration of ONO-1301 in an amount of 10-30 mg/kg.

2. JP2000-239188

JP2000-239188 discloses that a liquid agent or lotion comprising ONO-1301 in an amount of about from 0.01 to a few percent (w/v) is applied in amount of about from 0.1 to 1 mL per day for treatment of hair loss.

3. JP2000-86517

JP2000-86517 discloses that ONO-1301 is administered orally in an amount of 1 μg to 100 mg from once to a few times per day or parenterally in amount of 0.1 μg to 10 mg from once to a few times per day for hair loss.

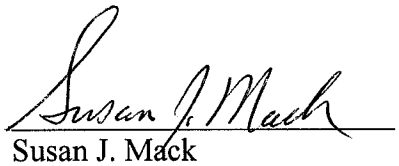
None of the cited references discloses that ONO-1301 is effective for any of the lung diseases recited in the present claims. Nor does any of the references teach that ONO-1301 accelerates production of HGF and VEGF. The effective administration amount disclosed in Hayashi and JP2000-86517 partially overlaps the effective dose for treating the lung diseases described in the present application. However, rats suffering from nephritis in Hayashi and animals suffering from hair loss or allodynia do not also necessarily suffer from any of the lung diseases recited in the present claims. Therefore, the present method claims are not anticipated, either expressly or inherently, by the cited references.

Accordingly, the Examiner is requested, respectfully, to reconsider and remove this rejection.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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Hepatocyte Growth Factor May Act as a Pulmotrophic Factor on Lung Regeneration after Acute Lung Injury*

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Hepatocyte growth factor (HGF) has been shown to have hepatotrophic and renotropic functions for regeneration of the liver and kidney through its mitogenic, motogenic, and morphogenic properties. To examine the involvement of HGF in lung regeneration after acute injury, we analyzed changes of HGF mRNA, HGF activity, and HGF receptors in the rat lung after lung insult and measured HGF concentration in sera of patients with various lung diseases. Following the onset of acute lung injury induced by intratracheal hydrochloride injection, a compensatory DNA synthesis occurred in the bronchial epithelium with a peak at 24 h and in the alveolar epithelium with a peak at 48 h. Expression of HGF mRNA in the rat lung remarkably increased only 3 h after the treatment and HGF activity in the lung also increased to about 3-fold at 6 h later. HGF receptors in the lung but not in the other noninjured organs were down-regulated 12 h later. These marked increases in HGF mRNA and HGF activity and the concomitant down-regulation of HGF receptor occurred before the marked compensatory DNA synthesis in bronchial and alveolar epithelial cells. HGF concentration in sera of patients with various lung diseases, as measured by radioimmunoassay, was much higher than that in healthy donors. These results suggest that HGF is newly produced in the lung after acute lung injury and may have a role in regeneration of the lung.

Hepatocyte growth factor (HGF)[1] was first detected in the plasma of partially hepatectomized rats as a potent mitogen for adult rat hepatocytes in primary culture (Ref. 1, and reviewed in Refs. 2 and 3) and was purified to homogeneity from rat platelets (4, 5) and subsequently from human plasma (6), rabbit serum (7), livers of CCl_4-administered rats (8), and conditioned medium of human lung fibroblasts (9). Mature heterodimer HGF derives from a single chain precursor of 728 amino acids by proteolytic processing and has four kringle domains in the α-chain and the serine protease-like domain in the β-chain (10–13). HGF targets a wide variety of epithelial cells and has multiple biological activities. HGF stimulates proliferation of various cells, including renal tubular epithelial cells (14), keratinocytes, melanocytes, and many other epithelial cells (9, 15), whereas it inhibits growth of hepatoma and melanoma cells (16–18). HGF strongly enhances cell motility of various epithelial cells as a motogen (15, 19–22) and induces branching tubule formation of Madin-Darby canine kidney cells in collagen gel as a morphogen (23). These pleiotrophic actions of HGF are mediated through high-affinity HGF receptors with a K_d value of 20–30 pM (24, 25). The high-affinity receptor is known to be a *c-met* proto-oncogene product which has the intracellular tyrosine kinase domain (26–28).

HGF functions as a hepatotrophic or renotropic factor for hepatic or renal regeneration, respectively. HGF mRNA and HGF activities markedly increase in the liver or plasma of rats after various liver insults (2, 3, 8, 29), and intravenously injected recombinant HGF markedly enhances liver regeneration in mice (30). Moreover, HGF mRNA and HGF activity also increases in the kidney after renal injury (31, 32).

On the other hand, since HGF receptors and *c-met* mRNA are present in the lung, the possibility that HGF participates in regeneration following lung injury would have to be given attention. To produce acute lung injury, we injected intratracheally hydrochloride (HCl injection) (33), as an experimental model of aspiration pneumonia which induces adult respiratory distress syndrome.

We described here evidence that both HGF mRNA and HGF activity markedly increased followed by marked down-regulation of the HGF receptor and subsequently by DNA synthesis of lung epithelial cells in the injured lung. Our findings lend support to the thesis that HGF may be a "pulmotrophic factor" for regeneration of an injured lung.

EXPERIMENTAL PROCEDURES

Animal Treatments—Adult male pathogen-free Wistar rats (200–250 g of body weight) were used in this study. To induce acute lung injury, we anesthetized the animals with ether and exposed trachea. An 18-gauge elaster needle was placed in the trachea and 0.1 M HCl 2 ml/kg body weight was injected into the lung (33).

Assay for Histological Change and Labeling Index—Fifty mg/kg body weight of 5-bromo-2′-deoxyuridine (BrdU, Sigma) was intraperitoneally injected into rats, and 1 h later these rats were anesthetized with ether, the lungs were removed, fixed with 70% ethanol for 12 h, and embedded in paraffin. For histological observation, 4-μm-thick sections were deparaffinized and stained with hematoxylin and eosin. To measure DNA synthesis, the cells undergoing DNA synthesis were immunohistochemically identified using anti-BrdU antibody, as described elsewhere (30). Enzyme reaction was initiated in buffer composed of 0.2 M Tris-HCl (pH 8.2), 250 μg/ml L-levamisole (Sigma), 0.01% new fuchsin (Merck), 0.01% sodium nitrite, 250 μg/ml naphthol AS-BI triphosphate (Sigma), and 0.25% *N,N*-dimethylformamide (34). The sections were washed with phosphate-buffered saline, fixed with 10% formalin, and counter-

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[1] The abbreviations used are: HGF, hepatocyte growth factor; BrdU, 5-bromo-2′-deoxyuridine; EGF, epidermal growth factor; TGF, transforming growth factor; KGF, keratinocyte growth factor; IGF, insulin-like growth factor.

stained with hematoxylin. The labeling index was counted in cells among more than 1000 nuclei of bronchial epithelia and alveolar septa, but granulocytes and lymphocytes were excluded from counting.

Preparation of Anti-rat HGF Antibodies—Recombinant rat HGF was purified from conditioned medium of Chinese hamster ovary (CHO) cells transfected with expression vector containing rat HGF cDNA (13).

To prepare polyclonal antibodies against rat HGF, purified rat HGF was injected into rabbit with Freund's adjuvant. IgGs were, respectively, purified from pre-immune serum and antiserum using protein-A Sepharose.

Northern Blot Analysis—Total RNA was isolated from the lung, using the acid-guanidium thiocyanate-phenol-chloroform method (35). Twenty μg of total RNA were applied to electrophoresis on 1.0% formaldehyde-agarose gels and transferred to a Hybond-N membrane filter (Amersham). The *Eco*RI fragment (1.4 kilobase pairs) of rat HGF cDNA (RBC-1 clone, Ref. 13) was labeled, using the Megaprime DNA labeling system (Amersham), according to the manufacturer's instruction. RBC-1 encodes the 5'-portion including the fourth kringle domain of α-chain, the entire β-chain of HGF, and a part of 3'-noncoding region. Hybridization was performed at 42 °C for 20 h in solution composed of 50% (v/v) formamide, 5 × SSPE (1 × SSPE consists of 0.15 M NaCl, 10 mM sodium phosphate buffer (pH 7.4) and 1 mM EDTA), 2 × Denhardt's solution (1 × Denhardt's solution consists of 0.02% Ficoll (Type 400, Pharmacia), 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin (Fraction V, Sigma)), 0.5% SDS, and 50 μg/ml salmon sperm DNA. The filter was washed with 0.2 × SSPE, 0.1% SDS for 15 min at 65 °C, then was dried and autoradiographed on Fuji x-ray film. The gel was stained with ethidium bromide to estimate the amount of loaded RNAs.

Partial Purification of HGF from the Lung—HGF was partially purified from the lung, as described elsewhere (1, 4, 8). Briefly, the lung was homogenized with Polytron in 4 volumes of buffer composed of 10 mM Tris-HCl (pH 7.5), 0.3 M NaCl, and 0.01% Tween 80 containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM monoiodoacetate, and 1 mM EDTA). After centrifugation at 105,000 × *g* for 1 h, the supernatant was dialyzed against 100 volumes of the same buffer, using cellulose tube with molecular cut off >M_r 30,000, and applied to a heparin-Sepharose column equilibrated with the same buffer. The column was washed with 2 volumes of the same buffer and then eluted with buffer containing 2 M NaCl. The eluate was dialyzed against buffer composed of 2 mM Hepes-NaOH (pH 7.2) and 20 mM NaCl, lyophilized, and then dissolved with H_2O. Samples used for HGF activity assay were passed through a 0.22-μm pore size filter (Millipore).

To determine whether stimulatory effect of sample prepared from rat lung on DNA synthesis of hepatocytes was attributable to HGF, test sample was incubated with pre-immune control IgG or anti-rat HGF IgG at 4 °C for 1 h and assayed for HGF activity.

Assay for HGF Activity—HGF activity was determined by measuring the stimulatory effect on DNA synthesis of rat hepatocytes in primary culture (4). Adult rat hepatocytes were isolated by the *in situ* collagenase perfusion method. The isolated cells were plated at a density of 6.25 × 10^4 cells, 0.5 ml, 2 cm^2 on 24-well plastic dish (Corning) coated with type I collagen and cultured in Williams' E medium containing 5% calf serum, 10^{-9} M insulin, and 10^{-9} M dexamethasone for 3 h. The medium was changed to serum-free Williams' E medium containing 10^{-9} M insulin, 10^{-9} M dexamethasone and 0.1 μg/ml aprotinin. After 20 h, test samples were added and the cells were cultured for 20 h. The cells were pulse-labeled with 1 μCi of ^{125}I-deoxyuridine for 6 h, and ^{125}I-deoxyuridine in the nuclei was measured in a γ-counter. One unit of HGF activity corresponds to the stimulatory activity which gives a half-maximal value of stimulatory effect on DNA synthesis of hepatocytes when 10 ng/ml EGF is added.

Assay for ^{125}I-HGF Binding to Plasma Membranes—Plasma membranes were prepared from lung, liver, kidney, and spleen of rats by density-gradient centrifugation on Percoll, as described elsewhere (36). Human recombinant HGF was radioiodinated by a chloramine-T method, as described elsewhere (37). The ^{125}I-HGF had a specific radioactivity of 70–160 mCi/mg of protein. Fifty μg of plasma membranes were incubated with 48 pM ^{125}I-HGF for 1 h at 10 °C, with or without 6.4 nM unlabeled HGF in 100 μl of binding buffer (Hanks' solution containing 10 mM Hepes, 2 mg/ml bovine serum albumin). Membranes were centrifuged for 10 min at 12,000 × *g* at 4 °C, resuspended with 10 ml of binding buffer, and transferred to fresh tubes. ^{125}I-HGF specifically bound to membranes was counted in a γ-counter. All binding experiments were done in triplicate.

Assay for HGF Concentration in Human Blood—HGF concentration in human sera was measured by sandwich radioimmunoassay using monoclonal anti-human HGF antibodies. Briefly, beads coated with anti-human HGF monoclonal antibodies were incubated with one-half-diluted human serum for 15 h at 25 °C. After washing with phosphate-buffered saline, the beads were incubated with ^{125}I-labeled anti-human HGF monoclonal antibodies for 4 h, and ^{125}I specifically bound to the beads was counted using a γ-counter. HGF concentrations in human sera can be precisely determined as low as 0.01 ng/ml.[2]

RESULTS

Histological Change after Induced Acute Lung Injury—To induce acute lung injury, 0.1 M HCl 2 ml/kg body weight was intratracheally administrated into the lung of each rat. In the intact lung, the intima of the bronchus consisted of simple columnar epithelia and that of the thin alveolar septa consisted of simple squamous epithelia (Fig. 1*A*). Three hours after HCl injection, hemorrhage in the submucosal region of the bronchus and in the alveolar space was obvious, and infiltration of polymorphonuclear leukocytes was present in several regions (Fig. 1*B*). Twelve hours later, there was histological evidence of the onset of acute lung injury: some pulmonary edema, de-epithelialization of the bronchus and alveoli, a neutrophilic cellular response in the submucosal layer of the bronchus and perivascular area, and hemorrhage accompanying the formation of fibrin-net-forming (Fig. 1*C*). Thereafter, hyaline degeneration, infiltration of macrophages, and epithelial proliferation was observed at 24–48 h, and mesenchymal cell proliferation and fibrous thickening of alveoli were present 3–7 days later (not shown). Thus, intratracheal administration of HCl produced a severe acute lung injury in rats.

DNA Synthesis in Bronchial Epithelia and Alveolar Septa following Lung Injury—To determine the time course of regenerative cell proliferation after acute lung injury, cells undergoing DNA synthesis were identified by the incorporation of BrdU followed by immunochemical staining (Fig. 2). In the untreated rat lung, there were few cells undergoing DNA synthesis (0.95% in bronchus and 1.42% in alveoli). However, there was a marked increase in the number of cells undergoing DNA synthesis following HCl treatment. The cells undergoing DNA synthesis were predominantly simple columnar cells in the bronchial epithelia (Fig. 2*A*) and alveolar type II epithelial cells in alveolar septa, characterized by a round nucleus and a comparatively small cytosol protruding into the alveolar space (Fig. 2*B*). Several endothelial cells with a small flat nucleus and macrophages in the alveolar space also underwent DNA synthesis, although the number of these cells undergoing DNA synthesis was much fewer than in epithelial cells.

The number of cells undergoing DNA synthesis in the bronchial epithelia and the alveolar septa remarkably increased from 24 h following HCl treatment (Fig. 3). Maximum values of the labeling index were 13.0% at 24 h in bronchial epithelia and 11.7% at 48 h in alveolar septa. Then, labeling indexes markedly decreased 72 h after HCl treatment and returned to almost normal levels within 1 week.

Change in HGF mRNA in Lung after HCl-induced Lung Injury—To determine whether or not HGF has a role in regeneration of the acutely injured lung, we examined HGF mRNA in the lung after HCl treatment (Fig. 4). HGF mRNA was detectable in the intact lung, and it markedly increased with a peak at only 3 h after HCl injection; the maximum level was 5-fold higher than normal. The high level of HGF mRNA expression in the lung continued for up to 12 h, and HGF mRNA levels reverted to normal at 24 h in two of three rats after HCl injection.

HGF Activity in Lung—HGF activity in the injured lung increased as early as 3 h after HCl injection and reached the maximum at 6 h (Fig. 5). The maximum value was 3–4-fold

[2] T. Kawamoto, H. Iwaki, K. Sekiguchi, and T. Nakamura, submitted for publication.

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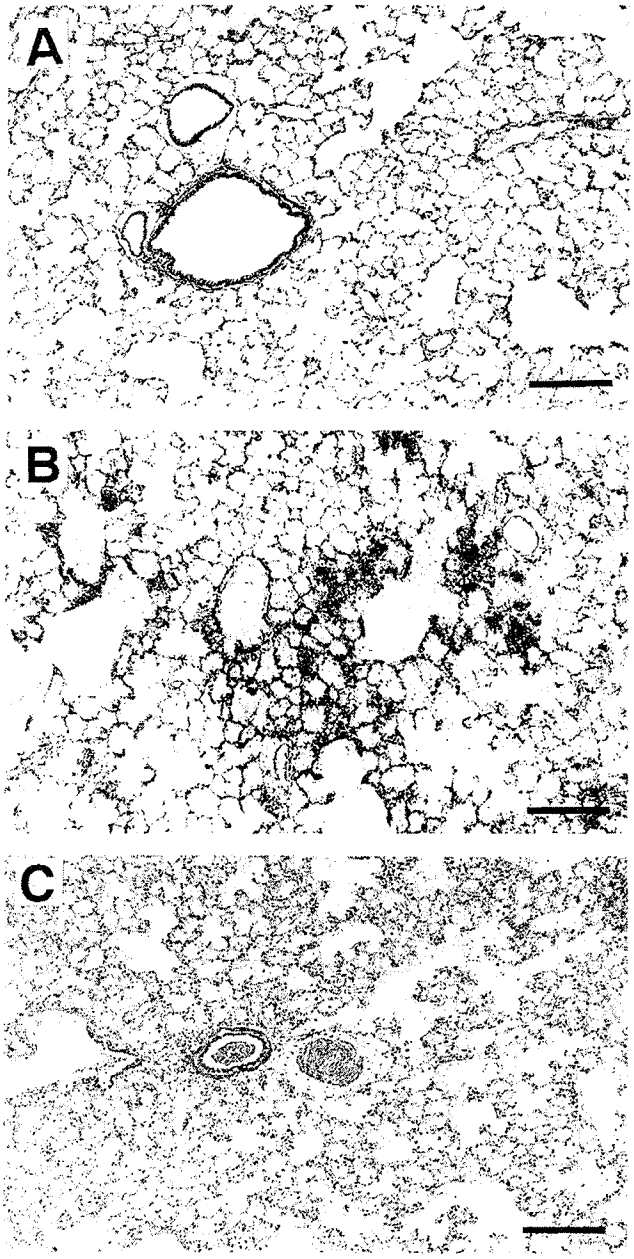

FIG. 1. **Histology of the lung after intratracheal HCl injection.** Acute lung injury was induced by intratracheal injection of 0.1 M HCl (2 ml/kg of body weight). The lungs were fixed with 70% ethanol and embedded in paraffin. Four-μm sections were deparaffinized and stained with hematoxylin and eosin. *A*, normal lung; *B*, 3 h after HCl treatment; *C*, 12 h after HCl treatment. *Bars* represent 200 μm.

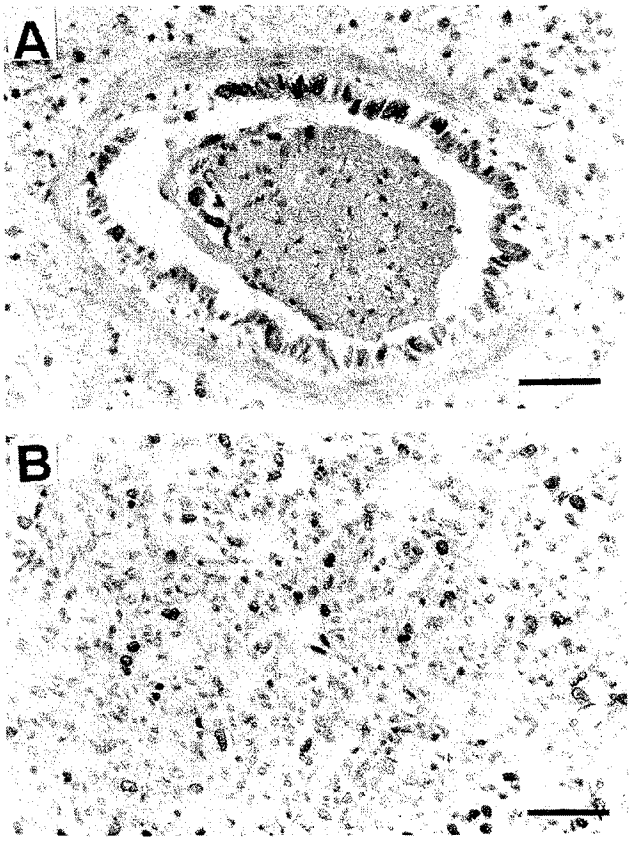

FIG. 2. **Distribution of cells undergoing DNA synthesis in the lung after HCl-induced acute lung injury.** These cells were immunohistochemically stained using anti-BrdU monoclonal antibody: *A*, bronchial epithelial cells at 24 h after HCl treatment; *B*, alveolar region at 48 h after HCl treatment. *Bars* represent 50 μm.

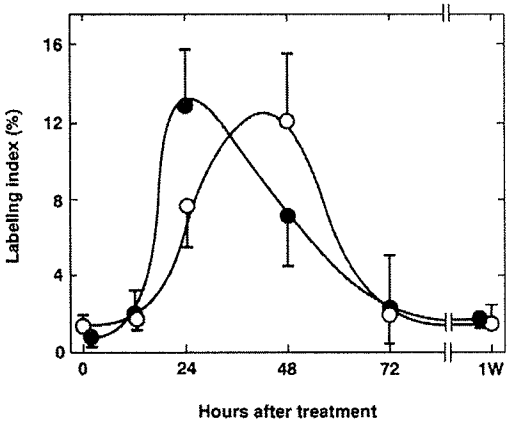

FIG. 3. **Time courses of DNA synthesis in bronchial and alveolar epithelial cells in rat lung after HCl-induced injury.** DNA synthesis was detected by BrdU uptake and following immunohistostaining using anti-BrdU monoclonal antibody. Labeling index (percent of cells undergoing DNA synthesis) was determined by counting labeled nuclei among more than 1000 nuclei of bronchial (●) and alveolar epithelial cells (○).

higher than that of the normal. HGF activity gradually decreased from 12 h after the treatment and reverted to normal within 48 h after the injection. Because HGF activity in the lung was almost completely abrogated by the addition of antiserum against recombinant rat HGF (Fig. 5) but not by preimmune serum (data not shown), HGF activity in the lung was attributable to HGF. These results suggested that HGF was newly synthesized in the injured lung following increase in HGF mRNA expression after the HCl-induced injury.

Change in Binding of ^{125}I-HGF to the Plasma Membranes —We reported that rapid down-regulation of cell surface HGF receptor was found only in the injured organs after liver or renal insult and not in intact organs (24, 25). Since down-regulation of growth factor receptors occurs immediately after ligand binding, these results suggest that HGF immediately produced at the injured site and other intact organs such as lung, spleen, and kidney (29, 38, 39) exerts biological activities specifically at the injured site, as a trophic factor for regeneration. Therefore, to examine whether HGF newly synthesized in the lung subjected to insult is involved in lung regeneration, we

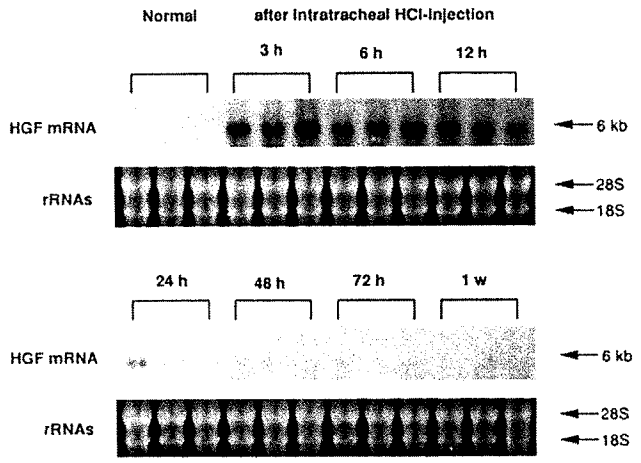

FIG. 4. **Northern blot analysis of HGF mRNA in rat lung after HCl-induced injury.** RNA was extracted from the lung of each rat at various times after HCl treatment. Twenty μg of total RNA were electrophoresed on 1.0% formaldehyde-agarose gel, transferred to Hybond-N nylon membrane filter, and hybridized with ^{32}P-labeled rat HGF cDNA: *lane 1–3*, normal; *lane 4–6*, 3 h after treatment with HCl; *lane 7–9*, 6 h after treatment with HCl; *lane 10–12*, 12 h after treatment with HCl; *lane 13–15*, 24 h after treatment with HCl; *lane 16–18*, 48 h after treatment with HCl; *lane 19–21*, 72 h after treatment with HCl; *lane 22–24*, 1 week after treatment with HCl. Ribosomal RNA (*rRNA*) are shown as an internal control after staining with ethidium bromide. *Arrows* indicate 28 and 18 S rRNAs.

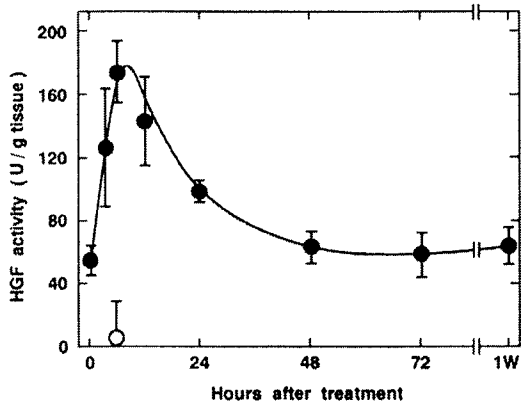

FIG. 5. **Change in HGF activity in lung after acute lung injury.** HGF was partially purified from the lung using heparin-Sepharose. HGF activity was determined by measuring stimulatory effects on DNA synthesis of adult rat hepatocyte in primary culture. Test samples were preincubated with (○) or without (●) anti-rat HGF IgG and assayed for HGF activity. One unit of HGF activity corresponds to the stimulatory effect on DNA synthesis of hepatocytes by adding 10 ng/ml EGF. Each value represents the mean ± S.D. of duplicate experiments on three rats in each group.

prepared plasma membranes from injured lungs and intact organs and analyzed HGF receptor. Specific binding of HGF was saturable at about 100 pM (not shown), and the amount of specific binding of HGF to plasma membranes from the lung decreased to 68% of that of normal lung at 3 h after HCl treatment, to 26% at 6 h, and almost completely disappeared during 12–48 h after HCl injection, as shown in Fig. 6A. The binding of ^{125}I-HGF recovered to 26% of normal levels at 72 h after the treatment and to 88% 1 week after the insult. Scatchard analysis of ^{125}I-HGF binding to plasma membranes prepared from normal and HCl-treated rat lung at 3 h after the injection revealed the K_d value to be 58 pM and the number of binding sites (B_{max}) was 1530 sites/μg of plasma membranes protein in the normal lung, whereas 1050 sites/μg of plasma membrane

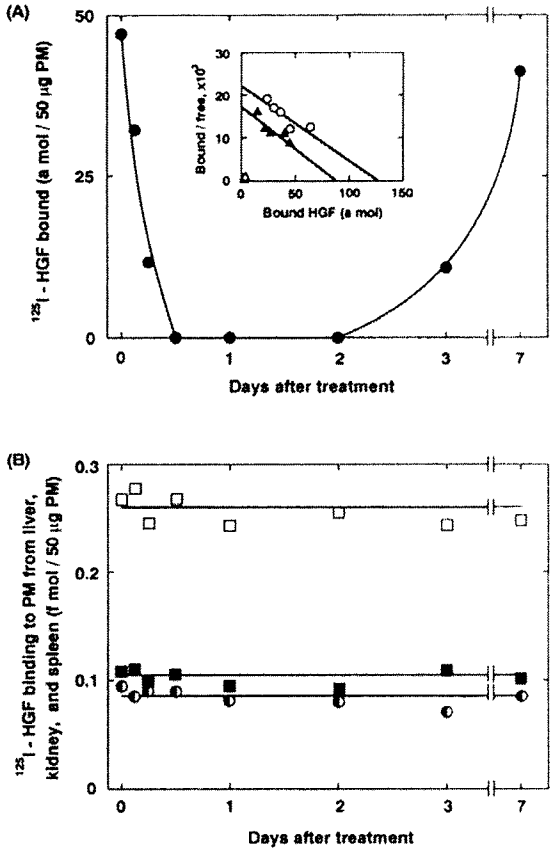

FIG. 6. **Change in binding of ^{125}I-HGF to the plasma membranes from lung, liver, kidney, and spleen after intratracheal HCl injection.** *A*, change of specific binding of ^{125}I-HGF to the plasma membranes (*PM*) from lung of each time point after HCl treatment (●). The *inset panel* shows a Scatchard analysis with plasma membranes from normal lung (○) and HCl-injured lung at 3 h (▲) and 12 h (△). *B*, changes of specific binding of ^{125}I-HGF to the plasma membranes from spleen (◐), kidney (■), and liver (□).

protein with K_d of 51 pM were present in lung 3 h after treatment (Fig. 6*A*, *inset*). Therefore, the decrease in specific binding of HGF after the induced injury was not due to change in affinity to the receptor, but rather to a decrease in the number of HGF receptors.

To determine whether down-regulation of HGF receptors was specific to the injured lung, we examined ^{125}I-HGF binding to plasma membranes purified from liver, kidney, and spleen after lung insult. As shown in Fig. 6*B*, there was no significant change in ^{125}I-HGF binding in the plasma membranes from these organs. These results clearly show that rapid down-regulation of the HGF receptor occurs specifically in the injured lung.

HGF Concentration in Sera of Patients with Lung Diseases—To examine changes in HGF levels in sera of patients with various lung diseases, we measured HGF concentration in sera from healthy donors and from patients with lung diseases, using a highly sensitive radioimmunoassay for HGF (Fig. 7). Elevated levels of HGF in sera of patients with lung diseases were found; HGF concentration in sera of 197 healthy donors was 0.33 ± 0.10 ng/ml, whereas HGF concentration in sera of 22 patients with lung diseases was 1.22 ± 1.01 ng/ml. Among patients with various lung diseases, HGF levels were 4.10 ng/ml in one with silicosis, 2.84 ng/ml in one with lung abscess, and in seven with pneumonia the level was 1.06–2.80 ng/ml.

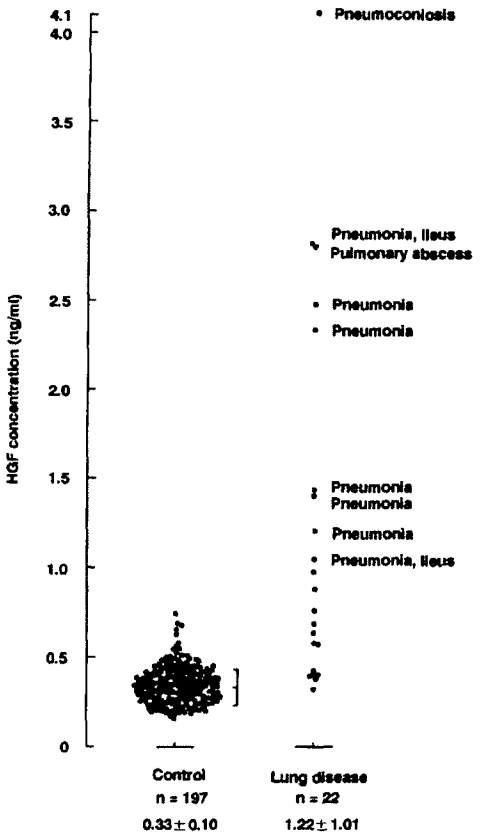

FIG. 7. **HGF concentration in sera of patients with lung diseases.** HGF concentrations in sera of healthy donors and patients with lung diseases were measured using sandwich radioimmunoassay.

DISCUSSION

As the lung is a respiratory organ, exposure to various exogeneous pathogens is inevitable. In alveolar injury, alveolar type I epithelial cells are predominantly damaged, de-epithelialization occurs, and alveolar type II cells actively proliferate to reconstruct the epithelium (40). In case of bronchial injury, remained bronchial epithelial cells, Clara cells, and basal cells are thought to proliferate and differentiate into the ciliated simple columnar cells and to compensate for the bronchial epithelia (41). Thus compensatory proliferation and subsequent differentiation of alveolar type II and bronchial epithelial cells seem to be essential for lung regeneration. Growth factors are considered to be involved in lung regeneration and several potent growth factors for bronchial and alveolar epithelial cells have been characterized. Proliferation of bronchial epithelial cells is stimulated by epidermal growth factor (EGF), transforming growth factor-α (TGF-α) (42), keratinocyte growth factor (KGF) (43) and insulin-like growth factor-I (IGF-I) (42), but it is inhibited by transforming growth factor-β (TGF-β) (44). DNA synthesis of alveolar type II cells is stimulated by insulin, EGF/TGFα, and acidic fibroblast growth factor (aFGF) (40, 45, 46). EGF enhances the formation of branching tubule structure in organ cultures of chick embryo (47), and an intravenous injection of EGF *in vivo* induces hyperplasia and metaplasia of bronchial epithelial cells (48).

HGF is synthesized and secreted by mesenchymal cells, such as macrophages, endothelial cells, and fibroblasts, and controls proliferation and morphogenesis of a broad spectrum of epithelial cells. It has recently been revealed that HGF is a potent mitogen for rat alveolar type II cells in primary culture (46). Therefore, we hypothesized that HGF may have important roles in regulating growth of lung epithelium and in regeneration of the lung, as a paracrine factor. In the present study, we obtained evidence which suggests that HGF has a role in regeneration of the lung following acute lung injury.

Following intratracheal HCl injection, HGF mRNA and HGF activity in lung remarkably increased as early as 3–6 h later, and the HGF receptor on the plasma membranes of lung was markedly down-regulated to an undetectable level 12 h later. Since the down-regulation of HGF receptor is probably caused by internalization of the HGF receptor following HGF binding, our results suggest that HGF exerts biological activities in the lung. Following marked changes in HGF mRNA, HGF activity, and the receptor in lung, there was marked increase in DNA synthesis of bronchial epithelial cells and alveolar epithelial cells during 24–48 h after the onset of lung injury. Thus the rapid and sequential induction of HGF mRNA and HGF activity that precede pulmonary epithelial cell proliferation strongly suggests that HGF may have a "trophic" role for the regeneration of lung following acute lung injury. Based on the pleiotrophic actions of HGF as mitogen, motogen (stimulation of cell motility), and morphogen, we propose that HGF may enhance lung regeneration through its multiple biological activities, *e.g.* not only by stimulating proliferation of bronchial and alveolar epithelial cells, but also by constructing normal tissue architecture of the bronchus and alveolus.

Although the HGF receptor on plasma membranes of the lung was markedly down-regulated, the HGF receptor in liver, kidney, and spleen did not change after lung injury. Thus HGF synthesized after lung injury specifically binds to the receptor in lung, but not to that in other intact organs. We reported that HGF mRNA is markedly induced in the intact lung following partial hepatectomy and unilateral nephrectomy in rats (39) and that the HGF receptor was specifically down-regulated in the injured organ but not in the intact lung (25). Our previous results suggested that HGF synthesized in the intact lung after hepatic or renal injury acts as a hepatotrophic or renotropic factor for regeneration, in an endocrine fashion. Therefore, taken together with present results, HGF synthesized in lung appears to contribute to the regeneration of the lung itself and also to regeneration of distal organs.

HGF concentrations in sera of patients with various hepatic and renal diseases were significantly higher than those in healthy donors. We reported direct evidence that HGF functions as an hepatotrophic and renotropic factor *in vivo*; administration of recombinant HGF into mice with hepatic or renal injury remarkably stimulated regeneration of the liver or kidney and prevented the onset of severe hepatic or renal dysfunction (30, 49). All these findings mean that HGF can be considered to treat subjects with hepatic and renal diseases, as well as being a diagnostic for these diseases. Since HGF concentration in sera of patients with various lung diseases were much higher than those in healthy donors, HGF seems to be involved in regeneration of lung also in human. Following acute or chronic lung injury, overgrowth of fibroblasts and overproduction of extracellular matrix often causes lung fibrosis which results in a decrease in respiratory functions. Since HGF is produced by mesenchymal cells and acts predominantly on epithelial cells, HGF is a putative "pulmotrophic factor" for regeneration of the lung, preventing onset of lung fibrosis. Our preliminary experiment using experimental animals has indeed proved that administration of HGF accelerate lung regeneration *in vivo* and plans are being designed to clinical administration of HGF to treat subjects with lung disorders.

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In vivo mitogenic action of HGF on lung epithelial cells: pulmotrophic role in lung regeneration

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Ohmichi, Hidenori, Kunio Matsumoto, and Toshikazu Nakamura. In vivo mitogenic action of HGF on lung epithelial cells: pulmotrophic role in lung regeneration. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L1031–L1039, 1996.—Hepatocyte growth factor (HGF) has mitogenic, morphogenic, and motogenic activities on epithelial cells and plays important roles in regeneration of the liver and the kidney. We previously found that the expression of HGF gene is rapidly induced in the lung after acute lung injury in experimental animals and that HGF levels are elevated in blood of patients with lung diseases. To search for a possible pulmotrophic function of HGF in lung regeneration, we examined the mitogenic activity of HGF on tracheal epithelial cells in vitro and evaluated the efficacy of HGF-administration on lung regeneration after acute lung injury in mice. HGF markedly stimulated proliferation and DNA synthesis of rat tracheal epithelial cells in primary culture in a dose-dependent manner. The intravenous injection of human recombinant HGF (10 $\mu g \cdot mouse^{-1} \cdot day^{-1}$) into mice with acute lung injury induced by the intratracheal infusion of 10 mM HCl stimulated DNA synthesis of airway epithelial cells to levels threefold higher than those in mice with no HGF-injections, but it did not stimulate DNA synthesis of alveolar epithelial cells. However, HGF injection at higher dose (100 $\mu g \cdot mouse^{-1} \cdot day^{-1}$) stimulated DNA synthesis of alveolar epithelial cells in vivo. These results indicate that HGF is a potent mitogen for airway epithelial cells and alveolar epithelial cells in vivo as well as in vitro. HGF may act as pulmotrophic factor responsible for airway and alveolar regeneration during lung regeneration after acute lung injury.

hepatocyte growth factor; acute lung injury; lung epithelial cell proliferation; bronchial regeneration; alveolar regeneration

HEPATOCYTE GROWTH FACTOR (HGF), originally considered to be a potent mitogen for mature hepatocytes, is a mesenchyme- or stroma-derived growth factor which targets a wide variety of cells (16). Initial purification and molecular cloning of HGF revealed that HGF is a heterodimeric molecule of a kringle-containing α-chain and a serine protease-like β-chain (18, 24, 25, 34). Characterization and cloning of scatter factor (3, 37), tumor cytotoxic factor (28), and lung fibroblast-derived epithelial mitogen (26) revealed that these molecules are identical with HGF, and these independent approaches revealed unique pleiotropic properties of HGF. HGF elicits mitogenic action for various types of normal epithelial cells (26), vascular endothelial cells (22), chondrocytes (33), and hematopoietic progenitor cells (11, 19). Motogenic activity of HGF to enhance motility of epithelial cells, acting as scatter factor, is closely linked to cell movement during epithelial wound healing and tumor invasion. It is noteworthy that HGF has a unique morphogenic activity for several types of cells, including epithelial branching tubulogenesis (8, 21, 31). Induction of branching tubulogenesis by HGF seems to be responsible for epithelial tubulogenesis during organogenesis of the kidney, mammary gland, liver, etc. (21, 27, 30, 31). HGF is thus considered to be a mediator of epithelial-mesenchymal interactions, essential tissue interaction during organogenesis.

On the basis of its potent mitogenic action for mature hepatocytes, HGF is a potent hepatotrophic factor for liver regeneration (7, 16). HGF mRNA and blood levels of HGF increase after various types of hepatic injuries (16, 40), and increased blood levels of HGF were noted in patients with hepatic diseases (29). More directly, the administration of recombinant HGF into animals with experimental liver injuries markedly accelerated liver regeneration in vivo (7). Similarly, the rapid increase of HGF mRNA in the kidney after induced renal injuries suggested a renotrophic role for HGF in renal regeneration (6, 23), and renotrophic function of HGF was recently demonstrated by administrating human recombinant HGF into experimental animals with acute renal injury (10, 17).

We have reported that HGF mRNA expression was rapidly induced in the lung after acute lung injury caused by intratracheal administration of HCl solution and that blood levels of HGF were elevated in patients with lung diseases (38). In vitro, HGF elicits mitogenic activity for alveolar type II epithelial cells (15). Taken together, we consider that HGF may act as a pulmotrophic factor for lung regeneration. In the present study, we obtained evidence that HGF is a potent mitogen for tracheal epithelial cells in primary culture and that the intravenously injected human recombinant HGF stimulated DNA synthesis of airway epithelial cells and alveolar type II epithelial cells in the lung after acute lung injury induced by intratracheal administration of HCl solution. Our results indicate that HGF exerts pulmotrophic actions for induction of regenerative DNA synthesis in airway and alveolar epithelial cells during lung regeneration, even when administrated intravenously.

MATERIALS AND METHODS

Primary culture of rat tracheal epithelial cells. Isolation and culture of tracheal epithelial cells were done as described elsewhere, but with minor modifications (4). Two rats (Nihon SLC, Shizuoka, Japan) were anesthetized with pentobarbital (50 mg/kg body wt), and the tracheas were surgically exposed. The proximal end of the trachea was intubated with the flanged tip of a micropipette and secured with surgical silk. The trachea was removed and washed with ice-cold MCDB-153 medium (Kyokuto, Tokyo, Japan). After washing the inside of trachea by perfusing it with the medium, the protease solution was infused through the tubing and the

distal end of trachea was closed. The trachea was expanded by infusing protease solution [MCDB-153 medium containing 1% of protease type XIV (Sigma), 100 U/ml of penicillin, and 0.1 mg/ml streptomycin], then incubated for 1 h at room temperature. To dissociate tracheal epithelia, medium present in the trachea was withdrawn and reinjected, using a syringe. The dissociated tracheal epithelial cells were suspended in 15 ml of MCDB-153 medium and centrifuged for 10 min at 150 *g*. Tracheal epithelial cells were resuspended in MCDB-153 containing 20% fetal bovine serum and then centrifuged for 5 min at 190 *g*. After the supernatant was removed, the cells were suspended in 13 ml of culture medium [MCDB-153 containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 7% $NaHCO_3$, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)·NaOH, pH 7.3, 10 μg/ml human transferrin, 5 μg/ml insulin, 5×10^{-7} M hydrocortisone, 5×10^{-7} M phosphoethanolamine and ethanolamine], and the cells were cultured at 37°C in 5% CO_2 and 95% air.

Measurement of DNA synthesis and cell growth. Tracheal epithelial cells were suspended in culture medium and plated on a 24-well plate (Corning). Cells were cultured for 24 h, the medium was changed to fresh culture medium, and cells were further cultured for 24 h. Growth factors were added, and cells were cultured for 24 h. The cells were pulse labeled with 1 μCi of ^{125}I-deoxyuridine for 20 h in thymidine-free culture medium containing growth factors. Cells were washed twice with phosphate-buffered saline (PBS) and then with ice-cold trichloroacetic acid. After solubilization with 1 M NaOH, ^{125}I-deoxyuridine in the nuclei was measured, using a gamma counter.

For the measurement of cell growth, tracheal epithelial cells were plated on a six-well plate at a density of 3.25×10^4 cells/cm^2 and cultured for 24 h. After the medium was changed to fresh medium [Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (DMEM-F12) 1:1 mixture containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 7% $NaHCO_3$, 15 mM HEPES·NaOH, pH 7.3, 3 mg/ml bovine serum albumin (BSA), 10 μg/ml human transferrin, 50 ng/ml insulin, 5×10^{-7} M hydrocortisone, 5×10^{-7} M phosphoethanolamine and ethanolamine], growth factors were added and the cells were cultured for 3 days. The number of cells was determined with the use of a hemacytometer.

Animal treatment. To induce acute lung injury, we used ether to anesthetize 8-wk-old ICR male mice (specific pathogen free, Nihon SLC) and surgically exposed the tracheas. An elaster needle (26 gauge) was placed in the trachea, and 0.01 M HCl (2 ml/kg) was injected into the lung through the needle.

When HGF was injected at 10 μg·mouse^{-1}·day^{-1}, mice were then given intravenously 5 μg of recombinant human HGF dissolved in 100 μl saline every 12 h from just before HCl injection, and animals were killed at 24 or 48 h after HCl injection. When HGF was injected at 100 μg·mouse^{-1}·day^{-1}, HGF was injected at 24, 30, 36, 48, 54, and 60 h after HCl treatment. A dose of HGF was 25 μg/mouse at 24, 30, 48, and 54 h and 50 μg/mouse at 36 and 60 h, respectively, after HCl injection. Control mice were given saline only. Each experimental group included three or four mice.

Assay for histological change and DNA synthesis in vivo. DNA synthesis of cells in tissues was measured by incorporation of 5-bromo-2'-deoxyuridine (BrdU) into nuclei and subsequent immunohistochemical staining using an anti-BrdU monoclonal antibody. BrdU dissolved in saline was intraperitoneally injected at 3 mg/mouse at 1 h before animals were killed. The excised tissues were fixed in 70% ethanol for 12 h and then embedded in paraffin after being dehydrated in a graded series of ethanol. The tissue sections were deparaffinized, endogenous peroxidase was inactivated in 0.3% H_2O_2 in methanol for 30 min and washed with PBS. DNA were denatured in 2 M HCl for 1 h, and sections were neutralized in 0.1 M borate buffer. To prevent the nonspecific binding of antibodies, the tissue sections were blocked in PBS containing 10% horse serum for 20 min. After washing with PBS, the sections were incubated for 1 h with anti-BrdU monoclonal antibody (Takara, Kyoto, Japan) diluted to 1:2,000 with PBS containing 5% horse serum. After washing with PBS, the tissues were incubated for 40 min with peroxidase-conjugated

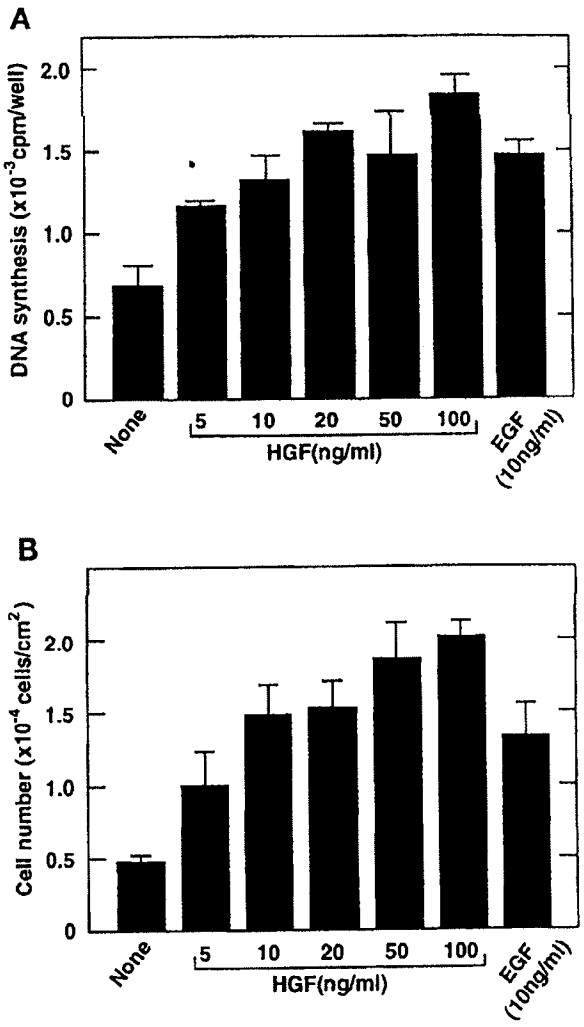

Fig. 1. Stimulatory effect of hepatocyte growth factor (HGF) on DNA synthesis (*A*) or proliferation (*B*) of rat tracheal epithelial cells in primary culture. *A*: bronchial epithelial cells were isolated from adult rat tracheas plated at a density of 3.25×10^3 cells/cm^2 on 24-well plastic dish. Growth factors were added 48 h after plating, and the cells were further cultured for 22 h. Cells were pulse-labeled with ^{125}I-deoxyuridine for 20 h, and radioactivity incorporated into nuclei was measured. EGF, epidermal growth factor. *B*: epithelial cells isolated from adult rat trachea were plated at a density of 3.25×10^3 cells/cm^2 and cultured for 24 h. Medium was changed to fresh medium, and growth factors were added. Cells were further cultured for 3 days, and no. of cells was determined with the use of a hemacytometer. Each value in *A* and *B* represents mean ± SD of triplicate measurements.

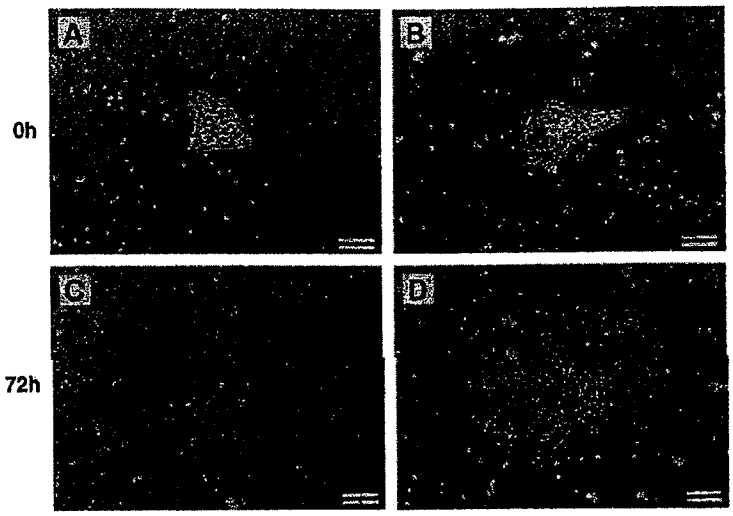

Fig. 2. Appearance of rat tracheal epithelial cells in primary culture, cultured in absence (*A* and *C*) or presence (*B* and *D*) of HGF for 3 days (72 h). Bronchial epithelial cells isolated from rat trachea were suspended in serum-free culture medium and plated onto culture dish at 3.25×10^3 cells/cm^2 (0 h). Twenty-four hours after plating, the medium was changed to fresh medium, HGF was added, and cells were cultured for 3 days in serum-free condition. Bars represent 25 μm.

rabbit anti-mouse immunoglobulin G diluted 1:200 with the same diluent. The sections were washed with modified PBS composed of 40 mM K_2HPO_4, 1 mM NaH_2PO_4, and 120 mM NaCl, and enzyme reaction was performed in modified PBS containing 0.1% H_2O_2 and 0.05% diaminobenzidine. All reactions were performed at room temperature. For histological observations, tissue sections were deparaffinized and stained with hematoxylin and eosin. The labeling indexes (percentage of BrdU-positive cells) in airway epithelial cells were determined by counting >1,000 nuclei, mainly in bronchiolar and terminal bronchiolar epithelia. The labeling indexes in alveolar epithelial cells represent the percentage of BrdU-positive alveolar type II epithelial cells among 10,000 nuclei in alveolar regions in the microscopic fields.

RESULTS

Mitogenic effect of HGF on tracheal epithelial cells in vitro. A previous report showed that HGF has mitogenic action for alveolar type II epithelial cells (15). To

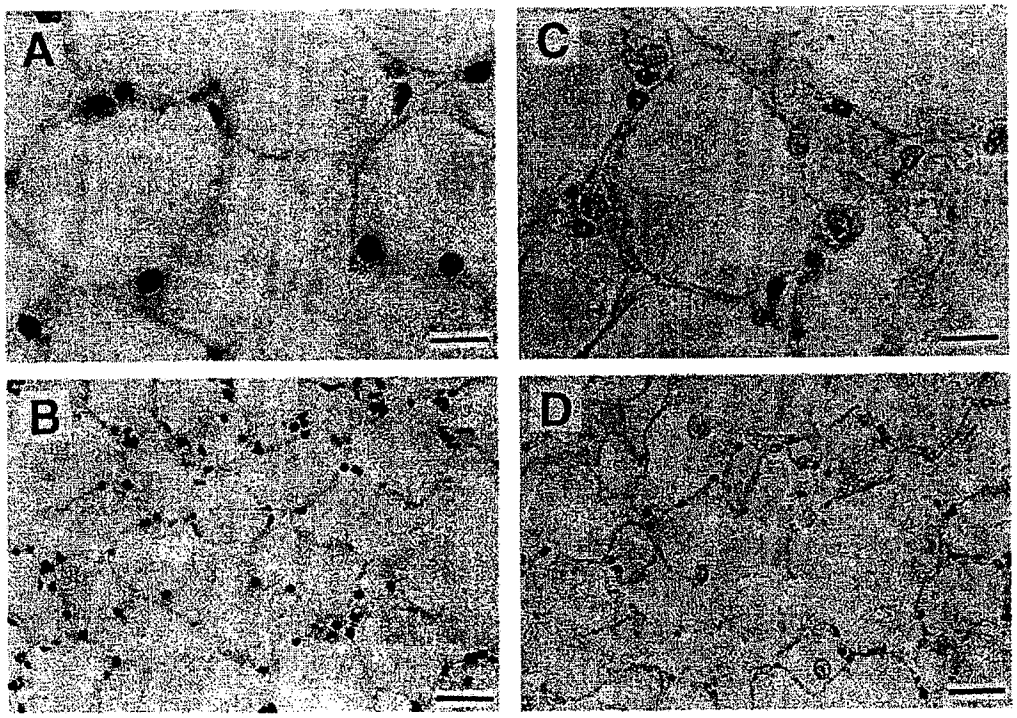

Fig. 3. Histological change in lung tissues of mice with acute lung injury induced by HCl injection. *A* and *B*: normal lung; *C* and *D*: 24 h after HCl treatment. Acute lung injury was induced by intratracheal injection of 0.01 M HCl (2 ml/kg body wt). Tissues were fixed in 70% ethanol and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin. Bars represent 10 μm in *A* and *C*, 25 μm in *B* and *D*, respectively.

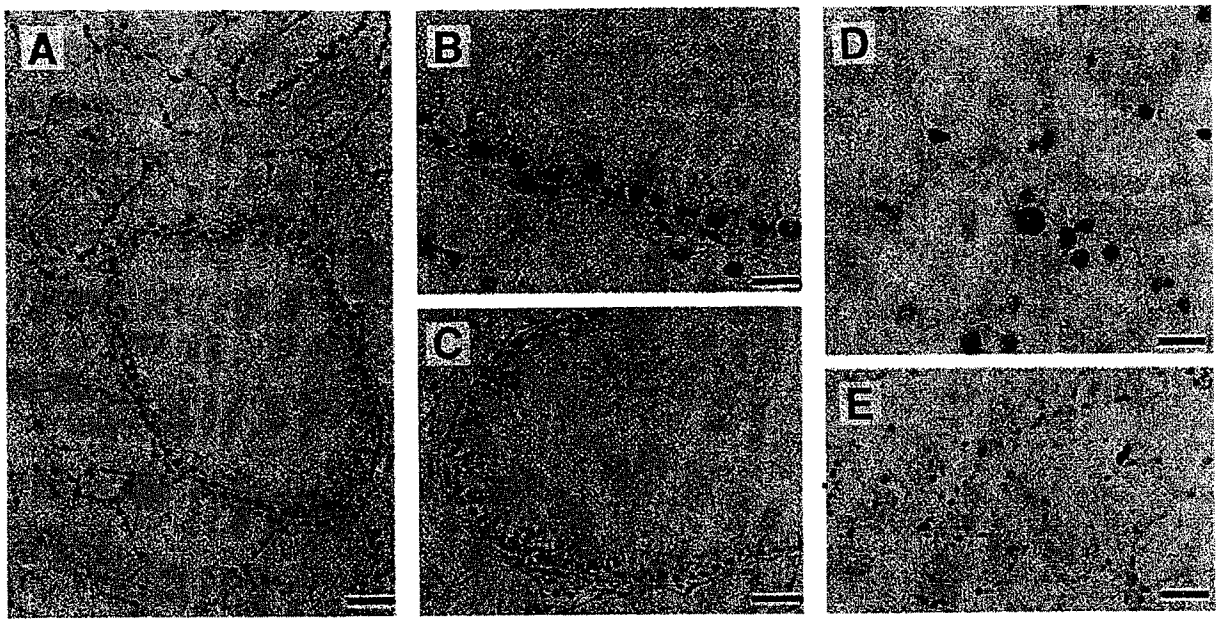

Fig. 4. Distribution of cells undergoing DNA synthesis in lung after HCl-induced acute lung injury. *A*: normal lung. *B* and *C*: around bronchiole at 24 h after HCl injection. *D* and *E*: alveolar region at 72 h after HCl injection. Acute lung injury was induced in mice by giving an intratracheal injection of HCl (2 ml/kg body wt). Cells undergoing DNA synthesis were detected by 5-bromo-2′-deoxyuridine (BrdU) incorporation and subsequent immunohistochemical staining using anti-BrdU monoclonal antibody. *B* and *D* represent airway and alveolar epithelial cells undergoing DNA synthesis. Bars represent 25 μm in *A*, *C*, and *E*; 10 μm in *B* and *D*.

know whether HGF has a mitogenic activity for airway epithelial cells, we examined the effect of HGF on DNA synthesis of rat tracheal epithelial cells in primary culture. The cells were cultured in serum-free conditions, and DNA synthesis was measured by ^{125}I-deoxyuridine incorporation. In the presence of HGF, ^{125}I-deoxyuridine incorporation was enhanced in a dose-dependent manner (Fig. 1*A*). The stimulatory effect was seen with 5 ng/ml of HGF, and a 2.5-fold maximum stimulation was seen with 100 ng/ml. The mitogenic effect of 10–50 ng/ml HGF is the same as seen with epidermal growth factor (EGF). Tsao et al. (35) also reported a mitogenic effect of HGF on normal human bronchial epithelial cells.

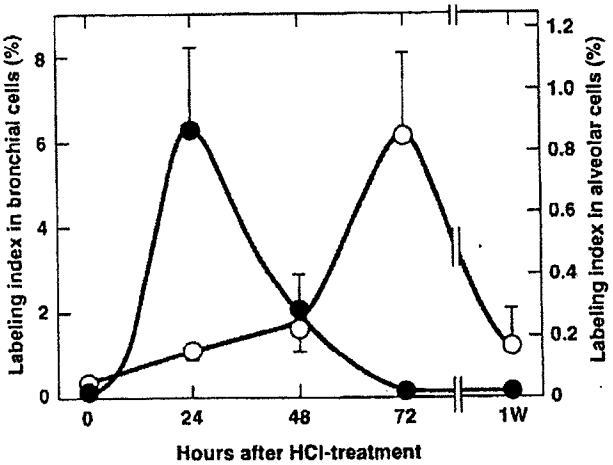

Fig. 5. Time courses of DNA synthesis in airway and alveolar type II epithelial cells after HCl-induced acute lung injury. After induction of acute lung injury by HCl injection, cells undergoing DNA synthesis were detected by BrdU incorporation and subsequent staining using anti-BrdU monoclonal antibody. Labeling indexes of airway (bronchial) epithelial cells (●) and alveolar type II epithelial cells (○) were determined by counting $>10^3$ cells for airway epithelial cells and $>10^4$ cells for alveolar epithelial cells. 1 W, 1 wk. Three mice were included in each experimental group, and each value represents mean ± SD.

Consistent with the mitogenic effect on tracheal epithelial cells, HGF stimulated proliferation of primary cultured tracheal epithelial cells during culture period of 3 days (Fig. 1*B*). A stimulatory effect of HGF on the proliferation of tracheal epithelial cells was seen at 5 ng/ml HGF, and the maximal fivefold stimulatory effect was seen at 50–100 ng/ml. The stimulatory effect of 10 ng/ml is the same as seen with 10 ng/ml EGF. Figure 2 shows the appearance of rat tracheal epithelial cells cultured in serum-free conditions in the presence or absence of HGF. During 3 days of culture, the number of tracheal epithelial cells did not increase in the absence of HGF (Fig. 2, *A* and *C*). In contrast, when cells were cultured in the presence of 20 ng/ml HGF, the size of the colony was markedly enlarged due to proliferation and enlargement of the cells (Fig. 2, *B* and *D*). Because HGF did not induce scattering of the tracheal epithelial cells, this factor did not elicit motogenic activity in these cells.

Lung regeneration after acute lung injury. During regeneration of the lung, basal cells and/or secretory

cells are thought to proliferate and differentiate into ciliated cells and goblet cells in airway epithelia (2, 9, 13), whereas alveolar type II epithelial cells proliferate and differentiate into type I and type II alveolar epithelial cells in alveolar epithelia (36). In the present study, acute lung injury was produced in mice by intratracheal administration of diluted HCl solution. The intact lung consisted of thin alveolar septa with an expansion of alveolar spaces (Fig. 3, *A* and *B*). Cuboidal cells on the surface of airway are airway epithelial cells, and cells with large nuclei protruding from alveolar epithelium are alveolar type II epithelial cells. Three hours after HCl administration, hemorrhage in the alveoli, destruction of alveolar septa, accumulation of edema fluid in alveolar spaces, and infiltration of polymorphonuclear leukocytes were observed (not shown). One day later, both expansion of alveolar spaces by disappearance of alveolar septa and pulmonary edema-like lung damage were observed, and alveolar macrophages, which are large cells free in the alveolar spaces, were seen (Fig. 3, *C* and *D*). On the other hand, dissociation of airway epithelia and edema fluid were observed in airway space (not shown). Two to three days later, hemorrhage and edema fluid in alveolar spaces disappeared, whereas the destroyed alveolar septa were still obvious (not shown).

To determine DNA synthesis of airway epithelial cells and alveolar type II epithelial cells during regeneration after acute lung injury, cells undergoing DNA synthesis were identified by incorporation of BrdU followed by immunochemical staining. In the intact lung, there were few cells undergoing DNA synthesis (Fig. 4*A*), the labeling index (% of cells undergoing DNA synthesis) of both airway epithelial cells and alveolar cells was <0.1%. Twenty-four hours after HCl administration, several airway epithelial cells were undergoing DNA synthesis (Fig. 4, *B* and *C*). Seventy-two hours after HCl treatment, cells undergoing DNA synthesis were distributed in alveolar regions and appeared to be mostly alveolar type II epithelial cells (Fig. 4, *D* and *E*). Most of labeled cells localized in alveolar regions have typical characteristics of alveolar type II epithelial cells, with relatively large cytoplasm at the alveolar surface and usually located in corners. In contrast, alveolar type I epithelial cells, endothelial cells with thin cellular structure, and infiltrating leukocytes with round nuclei and small cytoplasmic regions were not nuclear labeled at least within 72 h.

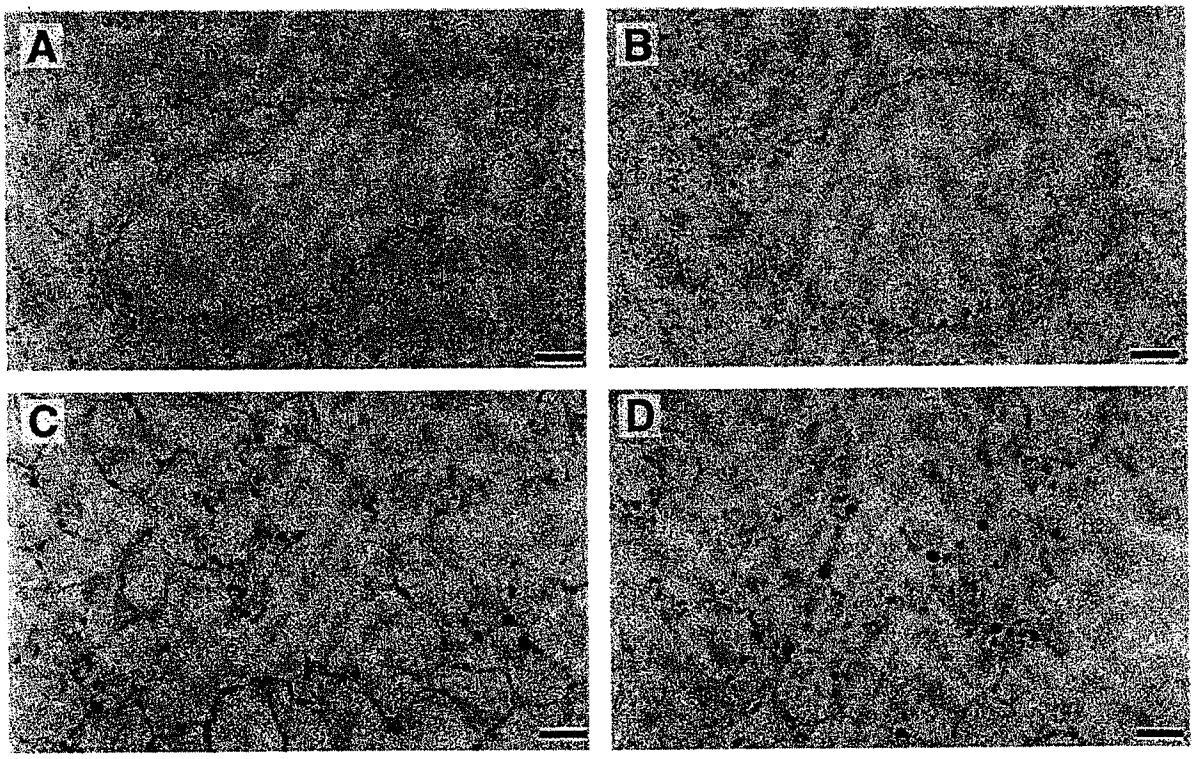

Fig. 6. Distribution of airway and alveolar type II epithelial cells undergoing DNA synthesis in mice given or not given HGF after acute lung injury caused by HCl. Mice were given saline alone (*A* and *C*) or HGF (10 μg·mouse^{-1}·day^{-1}, *B*; or 100 μg·mouse^{-1}·day^{-1}, *D*). *A* and *B*: area around bronchiole at 24 h after HCl injection. Mice were given saline alone (*A*) or HGF (5 μg/mouse) (*B*) at 0.5 and 12 h after HCl injection. *C* and *D*: alveolar region at 72 h after HCl injection. Mice were given saline alone (*C*) or HGF (*D*) at 24, 30, 36, 48, 54, and 60 h after HCl injection. A dose of HGF was 25 μg/mouse at 24, 30, 48, and 54 h or 50 μg/mouse at 36 and 60 h after HCl injection. Cells undergoing DNA synthesis were detected by BrdU incorporation and following immunohistochemical staining with anti-BrdU antibody. Bars represent 50 μm in *A* and *B*, 25 μm in *C* and *D*.

Figure 5 shows the time course of change in labeling index in airway epithelial cells and alveolar epithelial cells. Although the labeling indexes in both cell types were <0.1%, rapid induction of DNA synthesis occurred in airway epithelial cells, reaching the maximum value of 6%, then decreased to almost normal level at 72 h after HCl treatment. In alveolar type II epithelial cells, increase in the labeling index was not so marked within 48 h. However, the induction of DNA synthesis was thereafter obvious, and the labeling index reached a maximum level of 0.9% at 72 h. One week later, the labeling index decreased, but the level was still slightly higher than normal.

Mitogenic effect of recombinant HGF in vivo after lung injury. To examine whether HGF stimulates DNA synthesis of airway epithelial cells and alveolar type II epithelial cells in vivo, recombinant human HGF was injected intravenously into mice after the induction of acute lung injury. Although regenerative DNA synthesis occurred in several airway epithelial cells in saline-injected control mice (Fig. 6*A*), the administration of HGF (10 $\mu g \cdot mouse^{-1} \cdot day^{-1}$) increased the number of airway epithelial cells undergoing DNA synthesis (Fig. 6*B*). In contrast, DNA synthesis in alveolar type II epithelial cells, which usually are in corners of alveoli with relatively large cytoplasm and nuclei, were apparently not increased by HGF injection (Fig. 7*A*). The labeling index of airway epithelial cells was stimulated to a threefold higher level by HGF administration 24 h later, and the labeling index in HGF-injected mice was twofold higher than that of control mice 48 h later. However, there was no stimulatory effect of recombinant HGF on labeling index in alveolar type II epithelial cells at 24 and 48 h after HCl treatment (Fig. 7*A*).

Taking into account the possibility that the lack of mitogenic effect on alveolar type II epithelial cells was due to previous doses (10 $\mu g \cdot mouse^{-1} \cdot day^{-1}$) that were inadequate to exert biological effects in alveolar regions or that the inactivation of HGF by acidic condition was due to HCl remaining in alveolar space, a higher dose of HGF (100 $\mu g \cdot mouse^{-1} \cdot day^{-1}$) was used. HGF was injected intravenously from 1 day after HCl treatment, and the effect on DNA synthesis at 48 h and 72 h was measured (Fig. 6, *C* and *D*; Fig.7*B*). In this condition, HGF stimulated DNA synthesis of alveolar epithelial cells to a twofold higher level at 72 h but not at 48 h.

Because of mitogenic actions of HGF on various epithelial cells, including hepatocytes and renal tubular epithelial cells (5), it is likely that intravenously injected HGF may stimulate DNA synthesis of various types of cells in various organs. To determine whether mitogenic effects of HGF on lung injury are specific to the injured organ, the labeling index in liver and kidney was determined in mice with acute lung injury. After HCl treatment, there were few cells undergoing DNA synthesis in both liver and kidney, and labeling indexes in these cells were the same as in normal intact mice (not shown), indicating that the injury occurred specifically in the lung and that regenerative responses did not occur in liver and kidney. It should be emphasized that the intravenous injection of HGF did not induce DNA synthesis of cells in the liver and kidney (Fig. 8). The labeling index of cells in both organs remained at a normal level. In contrast, HGF injection strongly stimulated DNA synthesis of hepatocytes and renal tubular cells, when hepatic and renal injuries, respectively, occurred (Fig. 8, *C* and *D*). These results indicate that HGF stimulated DNA synthesis of cells in response to injuries in an organ-specific manner.

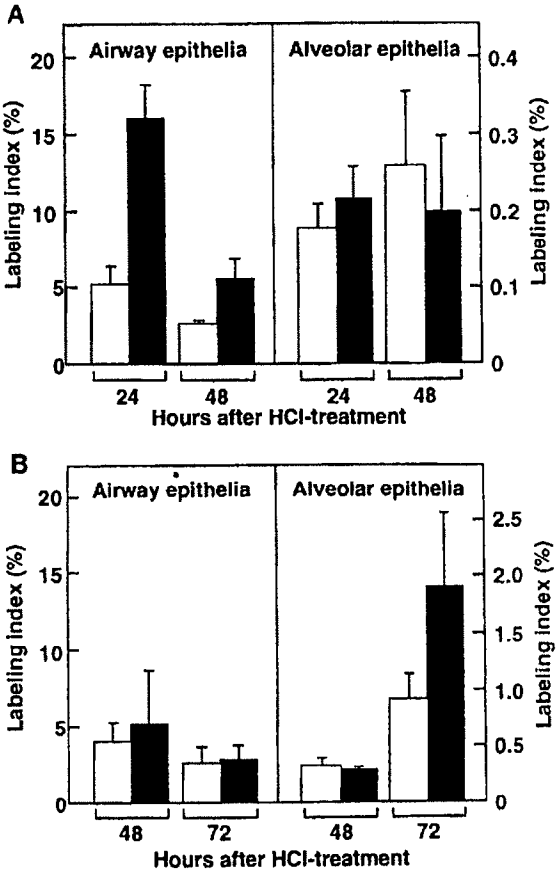

Fig. 7. Labeling index of lung epithelial cells in mice with or without HGF administration after acute lung injury. *A*: mice were injected with saline alone (open bars) or HGF (5 μg/mouse) (solid bars) at 0.5, 12, 24, and 36 h after HCl injection. *B*: mice were injected with saline alone (open bars) or HGF (solid bars) at 24, 30, 36, 48, 54, and 60 h after HCl. A dose of HGF was 25 μg/mouse at 24, 30, 48, and 56 h or 50 μg/mouse at 36 and 60 h after HCl injection. Cells undergoing DNA synthesis were detected by BrdU incorporation and subsequent immunohistochemical staining using anti-BrdU monoclonal antibody. Labeling indexes of airway epithelial cells and alveolar type II epithelial cells were determined by counting $>10^3$ cells for airway epithelial cells and $>10^4$ cells for alveolar epithelial cells. Three or four mice were included in each group, and each value represents mean ± SD.

DISCUSSION

Because of the lung's predominant function as a respiratory organ, exposure of lung epithelia to various exogenous pathogens is inevitable. In case of bronchial injury, basal cells and/or secretory cells dedifferentiate into multipotent progenitor cells and the airway epithelium is regenerated by differentiation of those cells (2,

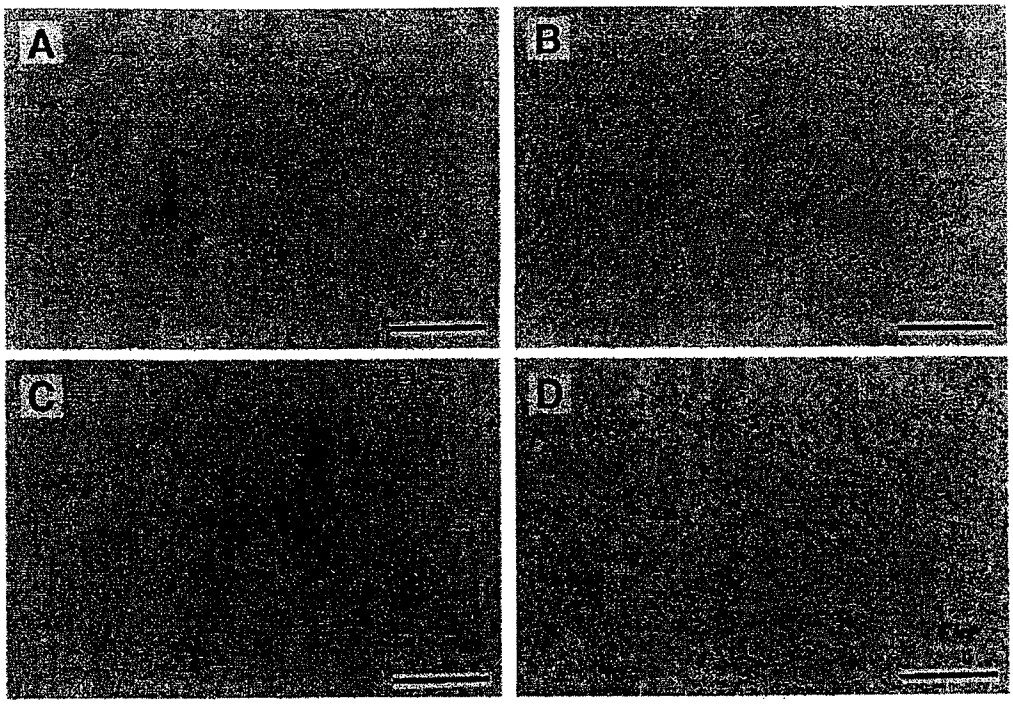

Fig. 8. Distribution of cells undergoing DNA synthesis in liver and kidney after HGF injection following induction of injury in lung and other organs. *A* and *B*: distribution of cells undergoing DNA synthesis in liver (*A*) and kidney (*B*) at 24 h after induction of lung injury. *C*: distribution of cells undergoing DNA synthesis in liver at 36 h after 30% partial hepatectomy. *D*: distribution of cells undergoing DNA synthesis in kidney at 72 h after induction of renal failure caused by $HgCl_2$ administration. Mice were injected with HGF (5 pg/mouse) at 0.5 and 12 h after HCl treatment (*A* and *B*), or at 0.5, 12, and 24 h after 30% partial hepatectomy (*C*), or at 48 h after $HgCl_2$ administration (*D*). Bars, 200 μm. Cells undergoing DNA synthesis were detected by BrdU incorporation and after immunohistochemical staining.

9, 13). In case of alveolar injury, alveolar type I epithelial cells are predominantly damaged, and alveolar type II epithelial cells proliferate and differentiate into alveolar type I epithelial cells (36). Thus compensatory proliferation of airway epithelial cells and alveolar type II cells is essential for regeneration of the lung. Several polypeptide growth factors stimulate proliferation of airway epithelial cells and alveolar type II epithelial cells. EGF, insulin-like growth factor-I (12), keratinocyte growth factor (1), and transforming growth factor-α (12) are mitogens for airway epithelial cells in vitro. On the other hand, acidic fibroblast growth factor (14), platelet-derived growth factor, EGF (36), and HGF (15) were reported to elicit mitogenic activity for alveolar type II epithelial cells in vitro. These growth factors are potential candidates as trophic factors for lung regeneration, i.e., "pulmotrophic factors"; however, potential roles of these growth factors and specificity in target cells during lung regeneration have gained increasing interest.

In the present study, we obtained evidence that HGF is a potent mitogen for tracheal epithelial cells in vitro and that intravenously injected HGF elicits mitogenic action for airway epithelial cells and alveolar type II epithelial cells in vivo. Together with previous findings that HGF has mitogenic activity for alveolar type II epithelial cells in vitro (15), HGF possibly has a pulmotrophic role for regeneration of lung epithelia after acute lung injury. We reported (38) that HGF mRNA induction rapidly occurs at 3–6 h with a marked downregulation of the HGF receptor, presumably due to receptor endocytosis after HGF binding, which occurred within 12 h after acute lung injury induced by HCl treatment. The results support the notion that HGF functions as a pulmotrophic factor for lung regeneration. The compensatory DNA synthesis seen in airway epithelial cells and alveolar epithelial cells occurred with distinct time courses, during the natural course of lung regeneration: a compensatory DNA synthesis was noted at 24 h in airway epithelial cells and at 72 h in alveolar epithelial cells. Moreover, it should be emphasized that the time at which exogenous HGF elicited potent mitogenic action coincided with the peak of DNA synthesis during the natural course of compensatory DNA synthesis. A potent stimulatory effect of HGF on DNA synthesis was seen at 24 and 72 h in airway and alveolar type II epithelial cells, respectively. The results indicate that the mitogenic activity of human recombinant HGF may not merely depend on the ligand concentration and that cellular competency to respond to HGF seems to be important for the regulation of HGF-dependent regeneration and DNA synthesis. Molecular mechanisms for the cellular competency should be determined, but we speculate that

the responsiveness to HGF may be regulated through modification of the receptor function (see below).

We previously showed that stromal cells of the lung such as endothelial cells and alveolar macrophage express HGF mRNA (39) and that HGF mRNA is expressed in mesenchymal cells, whereas c-Met/HGF receptor mRNA is expressed in epithelial cells in developing lung (Ref. 30; unpublished observations). Fibroblasts derived from embryonic lung produce a relatively large amount of HGF in vitro (3, 26, 28, 37). HGF acts as a hepatotrophic and renotrophic factor for hepatic and renal regeneration, at least through a paracrine mechanism. Therefore we speculate HGF may be involved in lung regeneration through a paracrine mechanism.

HGF is a stroma-derived factor which elicits mitogenic, motogenic, and morphogenic activities. It should be emphasized that the morphogenic activity of HGF to induce branching tubulogenesis is unique to this growth factor, which has important roles in the induction of branching tubular structures during development of organs, including kidney, liver, and lung (8, 21, 27). In our preliminary results, HGF enhanced enlargement and branching morphogenesis in organ culture of lung rudiments isolated from rat embryos 13 days postcoitus, whereas anti-sense HGF oligonucleotide inhibited branching morphogenesis in lung rudiments. Taken together, HGF may be involved in bronchial regeneration to reconstruct normal bronchial structures, through morphogenic activity to induce branching tubulogenesis, as well as its mitogenic activity to compensate damaged cells.

It is worth noting that the stimulatory action of HGF on DNA synthesis of cells occurs exclusively in an injured lung, not in intact organs such as the liver and kidney. We reported that the downregulation of HGF receptor, presumably due to receptor endocytosis after HGF binding, specifically occurs in injured lung, not in the intact organs (38). The downregulation of HGF receptor specific to the injured organ was also noted in injuries of other organs (32). In case of partial hepatectomy, a similar downregulation occurs specifically in the remnant liver, not in intact organs, such as the lung, kidney, and spleen, even though blood HGF levels increase after injury (5, 32). These findings suggest that HGF may exert activities in an injured organ and not in intact organs. This notion is consistent with the finding that long-term injection of HGF into normal rats led to no marked overgrowth and enlargement of various organs (unpublished observations). Concerning the injured organ specificity in the biological actions of HGF, the HGF receptor may be modified from nonfunctional to functional (capable of signal transducing) in response to the onset of organ injury. The putative conversion of the HGF receptor is speculative but does seem to relate to cell-cell interactions. Cell-cell contact modulates the response to HGF; DNA synthesis of hepatocytes is markedly stimulated by HGF at lower cell densities, but not in confluent cultures, and the downregulation of HGF receptor after HGF addition is also remarkable at lower density and does not occur in confluent culture (20). These observations suggest that cell-cell contact regulates function of the HGF receptor, and lung injury is likely to destroy this cell-cell contact through adherence junctions and/or gap junctions.

HGF, originally identified as a potent mitogen for mature hepatocytes, is a predominantly hepatotrophic factor for liver regeneration and a renotrophic factor for renal regeneration. The intravenous injection of recombinant HGF into experimental animals prevents the onset of severe hepatic or renal dysfunction and enhances regeneration. Thus HGF is expected to become a potent therapeutic tool for treating injuries and diseases. Our present study also implicates therapeutic potential for the treatment of pulmonary injuries and diseases. Finally, taking together our previous and present studies, we propose that HGF may function as an organotrophic factor which targets specific types of cells in various tissues and organs for purposes of regeneration.

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Enhanced Expression of Hepatocyte Growth Factor by Pulmonary Ischemia–Reperfusion Injury in the Rat

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Hepatocyte growth factor (HGF) has recently been noted to function as a pulmotrophic factor for lung regeneration. The present study was conducted to determine if HGF would be induced in a rat model of pulmonary ischemia–reperfusion (IR) injury, which was established by occlusion of the left lung, and to examine the significance of HGF in subsequent lung repair. The sham-operated rats underwent simple thoracotomy in which the lung was not clamped. We measured the plasma and the tissue levels of HGF by enzyme-linked immunosorbent assays, and the expression of HGF mRNA by Northern blotting. The plasma HGF level was markedly elevated after pulmonary ischemia and reached the peak value on the third postoperative day, being 5-fold higher than that of the sham-operated rats. HGF mRNA expression and the tissue HGF levels were augmented twofold in the ischemic reperfused lung. Immunohistochemical analysis revealed that the infiltrating alveolar macrophages were intensely stained for HGF. DNA synthesis of alveolar epithelial cells, as identified by proliferating cell nuclear antigen (PCNA) staining, was 3-fold higher in the reperfused lung than in the sham-operated lung. Notably, HGF-neutralizing treatment with an anti-HGF antibody reduced DNA synthesis of alveolar epithelial cells in the reperfused lung and aggravated lung injury. This study shows that HGF was induced in the ischemic reperfused lung and may play an important role in regeneration of an injured lung after pulmonary IR.

Hepatocyte growth factor (HGF) was initially detected in the plasma of partially hepatectomized rats as a potent mitogen for mature hepatocytes in primary culture, and was purified to homogeneity from rat platelets (1–3). HGF is a heterodimer molecule composed of 69-kD α-subunit and 34-kD β-subunit, and contains four kringle domains in the α-chain (4, 5). In addition to mitogenic activity for hepatocytes, recent studies have shown that HGF is a pleiotropic factor that is produced by mesenchymal cells and acts on a wide variety of epithelial cells, including renal tubular cells, melanocytes, and keratinocytes (6, 7). HGF strongly enhances cell motility of various epithelial cells as a motogen, and induces branching tubule formation as a morphogen (7–9). HGF is now recognized as a humoral mediator of epithelial–mesenchymal interaction in tissue regeneration (7). HGF messenger RNA (mRNA) and HGF activity markedly increase in the liver of rats after various liver insults (10–12), and intravenously injected recombinant HGF markedly enhances liver regeneration in mice (12). HGF mRNA and HGF activity also increase in the kidney after renal injury (13). Thus, HGF has been recently reported to function as a hepatotrophic or renotropic factor for hepatic or renal regeneration, respectively.

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More recently, HGF has been noted to function as a pulmotrophic factor for regeneration of an injured lung. In the injured lung, the alveolar type II cells contribute to regenerating the alveolar structure as progenitor cells in the repair process (14). *In vitro*, HGF stimulates DNA synthesis of the alveolar type II cells (15, 16). HGF mRNA expression was rapidly induced and DNA synthesis of the alveolar type II cells was increased in the lung after acute lung injury caused by intratracheal administration of hydrochloride (HCl) solution (17). Furthermore, intravenous and intratracheal administration of recombinant HGF stimulates DNA synthesis of alveolar type II cells in the rat lung after acute lung injury (18, 19). Recent studies have reported that HGF levels are elevated in the serum and bronchoalveolar lavage fluid of patients with inflammatory lung diseases, and in the pulmonary edema fluid of patients with acute lung injury (17, 20–22). These findings prompted us to investigate the role of HGF in lung injury and subsequent regeneration after thoracic surgery.

We have recently demonstrated that serum HGF levels are markedly elevated during the early period after transthoracic esophagectomy, which is accompanied by lung compression and collapse under unilateral ventilation (23). From the view of HGF involvement in tissue regeneration, it is tempting to speculate that pulmonary ischemia–reperfusion (IR) caused lung injury, which in turn enhanced the expression of HGF for lung regeneration. In spite of recent increasing information about the activities of HGF, no study has yet been conducted to examine the relationship between pulmonary IR injury and HGF expression. In this study, we investigated the expression of HGF at the peptide and mRNA levels using a rat model of pulmonary IR injury, which was established by occlusion of the left lung. Furthermore, we administered an anti-HGF antibody to rats of pulmonary IR, and examined the influence of HGF neutralization on DNA synthesis of alveolar epithelial cells to assess whether HGF plays an important role in regeneration of an injured lung after pulmonary IR.

METHODS

Animals

Male Wistar rats (Charles River Japan, Inc., Yokohama, Japan) weighing 250 to 300 g were used in this study. Animals were housed for at least 7 d under controlled light/dark conditions in which the light period was from 8:00 A.M. to 8:00 P.M. They were allowed free access to ordinary pellet diet and tap water *ad libitum*. All the experimental procedures were reviewed and approved by the Animal Care and Use Committee of the Nara Medical University.

Pulmonary IR Model and Experimental Groups

Rats were anesthetized by intraperitoneal administration of 50 mg/kg sodium pentobarbital and tracheostomized with a 14-gauge angiocatheter. The lungs were ventilated with 100% O_2 at a rate of 60 breaths/min with a tidal volume of 6 ml/kg body weight using a Harvard-type animal respirator (Harvard rodent ventilator Model 683; Harvard Apparatus Co., Millis, MA). A left thoracotomy was performed at the fifth intercostal space, and then the left pulmonary hilus

was exposed and clamped at its base using a vascular clip followed by placement of the lung and clip in the pleural cavity for 1 h. The tidal volume was reduced to 3 ml/kg only during clamping of the left hilus. After surgically implemented ischemia, the left lung was reinflated and reperfused by simultaneous releasing of clamping of the left hilus and increasing the tidal volume to 6 ml/kg. After closing the thorax in two layers, the rats were set free to breathe room air. The sham-operated rats underwent simple left thoracotomy in which the left lung was not clamped, and the control rats were only ventilated without thoracotomy. The sham-operated and control lungs were ventilated with a tidal volume of 6 ml/kg for 1 h.

Measurement of HGF in Plasma

Blood samples were serially obtained from the tail vein of five rats in each group at five timed intervals: before surgery, and on the first, third, fifth, and the seventh postoperative days (PODs). Blood samples were collected in pyrogen-free tubes containing ethylenediaminetetraacetic acid (EDTA), centrifuged at 1,000 × *g* for 10 min, and the resultant plasma was stored at −80° C until the plasma HGF assay. HGF concentrations in plasma were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Institute of Immunology, Tokyo, Japan) according to the protocol supplied by the manufacturer. The minimum detectable level of HGF with the ELISA kits was found to be 0.2 ng/ml. All the samples were assayed at least in duplicate.

Measurement of HGF in Tissue Extracts

The liver and lungs of five rats at each stated time point were excised after gentle perfusion with saline through the right ventricle to remove the blood thoroughly from these organs. During the perfusion, we confirmed that the color of the liver tissue changed from dark red to light red as the blood was successfully removed from the systemic circulation as well as from the pulmonary circulation. The liver and lung tissues were frozen instantly in liquid nitrogen. Tissue extracts were prepared as previously reported (24). Briefly, the liver and lung tissues were homogenized with Polytron (Kinematica AG, Littau, Switzerland) in 4 volumes of buffer composed of 20 mM Tris–HCl (pH 7.5), 2 M NaCl, and 0.01% Tween 80 containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA). The homogenate was centrifuged at 15,000 rpm for 30 min and the resultant supernatant was used as tissue extract. HGF concentrations in tissue extracts were measured using commercially available ELISA kits (Institute of Immunology) according to the protocol supplied by the manufacturer. All the samples were assayed at least in duplicate.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from the liver and lung tissues, using the acid guanidium thiocyanate–phenol–chloroform method (25). Ten micrograms of total RNA was subjected to electrophoresis on a 1% agarose-formaldehyde denaturing gel with 1× MOPS (4-morpholinepropanesulfonic acid) buffer and transferred to a Hybond-N nylon membrane filter (Amersham). Hybridization was performed at 42° C for 20 h in a solution composed of 50% (vol/vol) formamide, 5× SSPE (0.75 M NaCl, 50 mM sodium phosphate buffer, and 5 mM EDTA), 2× Denhardt's solution (1× Denhardt's solution consists of 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), and 100 μg/ml salmon sperm DNA. The *Xba*I–*Bam*HI fragment (2.6 kilobase pairs) of pBS rat was used as the hybridization probe and was labeled with [^{32}P]dCTP using a Random Primer Labeling Kit (Amersham), according to the manufacturer's instructions. The *Xba*I–*Bam*HI fragment of pBS rat included the entire open reading frame of rat HGF cDNA (26). After hybridization, the filter was washed twice with 2× SSPE/0.5% SDS for 15 min at room temperature followed by twice washing with 0.2× SSPE/0.1% SDS for 30 min at 65° C, and then dried and autoradiographed. The same filter was later hybridized to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as an internal reference for the quantification of total mRNA. Densitometric analysis was performed with a Bio-Image Analyzer (Fuji Photo Film, Tokyo, Japan).

Quantitative Assessment of Lung Injury

Lung histology. Lung tissues obtained from five rats at each time point were fixed with 70% ethanol at 4° C for 12 h, and then embedded in paraffin. Each section was cut to a thickness of 4 μm and was stained with hematoxylin and eosin. For the quantitative histopathological analysis of lung injury, a scoring system was used on the basis of the following four criteria: (*1*) destruction of the alveolar architecture; (*2*) neutrophil infiltration; (*3*) capillary congestion; and (*4*) pulmonary edema. Each criterion was graded on a scale of 0 to 3: 0, absent; 1, mild; 2, moderate; and 3, severe, and a total score was calculated for each specimen. To prevent observer's bias, all specimens were randomly numbered and evaluated in a blinded fashion by a pathologist. The mean of the scores was calculated for each time point and considered as the lung injury score.

Myeloperoxidase (MPO) assay. The extent of neutrophil sequestration was quantified by measuring MPO activity in the lung tissue according to the method described by Mullane and colleagues (27). Briefly, frozen lung tissue was homogenized in hexadecyltrimethyl ammonium bromide phosphate buffer (0.5%, pH 6.0), and the homogenate was centrifuged at 40,000 × *g* for 15 min. Fifty microliters of supernatant was added to 1.4 ml of 50 mM phosphate buffer (pH 6.0) containing 0.001% hydrogen peroxide, and then combined with 50 μl of 30 mM *o*-dianisidine hydrochloride (Sigma). MPO activity was assayed by measuring the change in spectrophotometric absorbance (optical density, O.D.) at 460 nm for 1 min and expressed as O.D./g of lung tissue.

Wet-to-dry weight ratio. Each harvested lung sample was weighed for the determination of wet weight and then dried in an oven at 70° C for 48 h. The dry tissue weight was determined and the lung wet-to-dry (W/D) ratio was calculated to assess pulmonary edema.

Immunohistochemistry

Lung tissues were fixed with 70% ethanol at 4° C for 12 h, dehydrated, and embedded in paraffin. Each section was cut to a thickness of 4 μm and stained for endogenous HGF, with an avidin–biotin-coupling (ABC) immunoperoxidase technique using a commercial kit (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA), according to the instructions of the manufacturer. After deparaffinization, the sections were incubated at 4° C overnight with a rabbit immunoglobulin G (IgG) against rat HGF (1:1,000) (Institute of Immunology) for the primary reaction. After three washes with phosphate-buffered saline (PBS), the sections were further reacted with biotinylated goat anti-rabbit IgG at room temperature for 2 h. Immunostaining for HGF was visualized with 3,3′-diaminobenzidine tetrahydrochloride containing 0.01% hydrogen peroxide. To identify alveolar macrophages, mouse IgG against rat macrophage (ED-1) (1:500) (Serotec, Raleigh, NC) was applied on the dewaxed sections for the primary reactions. Following washing, the sections were incubated with a secondary rabbit anti-mouse IgG at room temperature for 30 min. After incubation with the alkaline phosphatase-conjugated anti-alkaline phosphatase (APAAP) complex (Dako Japan, Kyoto, Japan), alkaline phosphatase (AP) activity was developed in red with the New Fuchsin AP substrate system (Dako Japan) according to the instructions of the manufacturer.

Measurement of DNA Synthesis in the Lung

To determine the regenerative cell proliferation after pulmonary IR injury, cells undergoing DNA synthesis were identified by immunohistochemical staining for proliferative cellular nuclear antigen (PCNA). PCNA staining was performed on the dewaxed sections, using a commercial kit with a monoclonal antibody against PCNA (clone: PC-10) (EPOS kit; Dako Japan). The PCNA labeling index was determined by counting more than 1,000 nuclei of alveolar epithelial cells in randomly selected microscopic fields.

Anti-HGF Antibody Treatment

To assess the mitogenic effect of endogenous HGF on alveolar epithelial cells of an injured lung, another 12 rats with pulmonary IR were randomly divided into two groups and injected intraperitoneally with the neutralizing anti-rat HGF rabbit IgG (n = 6) or normal rabbit IgG (n = 6) at 1, 12, 24, and 36 h after declamping of the left hilus. A dose of an anti-HGF antibody was 400 μg/rat at 1 h, and 200 μg/rat at 12, 24, and 36 h, respectively, after declamping. An anti-rat HGF IgG was prepared as previously described (28). These rats were sacrificed at 48 h after declamping of the left hilus, and the PCNA labeling index was determined as described above.

Statistical Analysis

The results are expressed as the mean ± standard deviation. The means of different groups were compared using a one-way analysis of variance. Statistical analysis was performed with the unpaired Student's *t* test. A p value < 0.05 was considered significant.

RESULTS

Histological Changes after Pulmonary IR

The 4-μm-thick sections of lung tissues were stained with hematoxylin and eosin, and the visual time course of the histo-

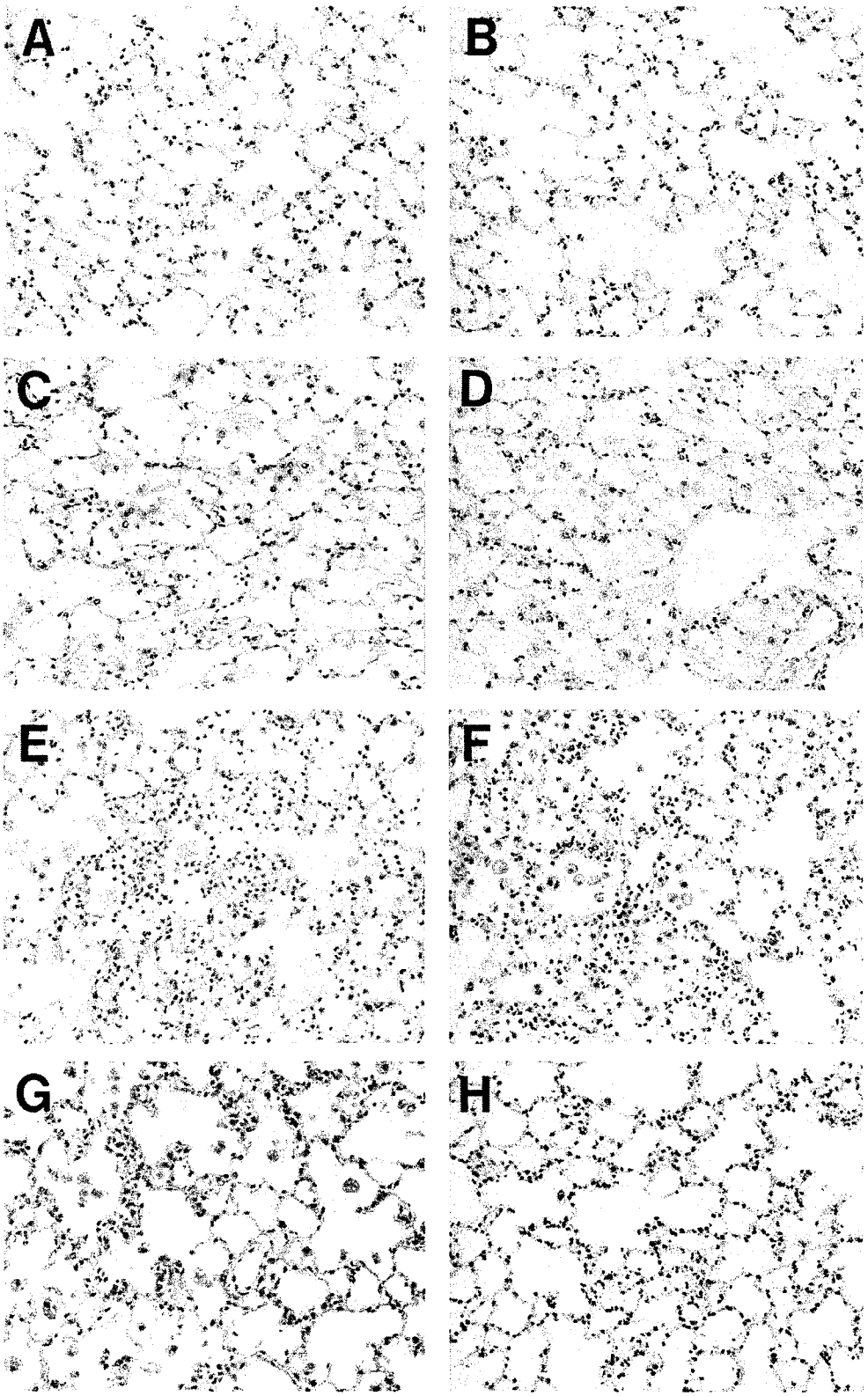

Figure 1. Histological changes in lung tissues after pulmonary IR. (*A*) Normal lung; (*B*) control left lung on POD 1; (*C*) sham-operated left lung on POD 1; (*D*) nonischemic opposite right lung of rats with left pulmonary IR on POD 1; (*E–H*) ischemic-reperfused left lung of rats with pulmonary IR. (*A*) In normal lung, the intima of the thin alveolar septa consist of simple squamous epithelia. (*B*) In the control left lung, minimal morphological changes were detected. (*C*) In the sham-operated left lung, moderate infiltration of neutrophils and macrophages into the alveoli with focal fibrinous exudate were observed. (*D*) In the nonischemic opposite right lung of rats with left pulmonary IR, moderate infiltration of macrophages into the alveoli and mild hyperemia of the alveolar septa were observed. Extensive edema and fibrinous exudates in alveolar lumina were also obvious. (*E*) In the ischemic reperfused left lung of rats with pulmonary IR on POD 1, neutrophils markedly infiltrated into the alveolar septa and alveoli, and moderate infiltration of macrophages into the alveoli with focal fibrinous exudate was observed as well as severe destruction of the alveolar architecture. (*F*) In the reperfused left lung on POD 3, severe infiltration of macrophages and increased alveolar epithelial cell proliferation were observed. (*G*) In the reperfused left lung on POD 5, mild infiltration of macrophages was observed. (*H*) In the reperfused left lung on POD 7, alveolar epithelial cell proliferation had decreased, and few macrophages into the alveoli were observed (hematoxylin–eosin stain; original magnification: ×120).

pathological findings are shown in Figure 1. In normal lung, the intima of thin alveolar septa consist of simple squamous epithelia (Figure 1A). In the control left lung on POD 1, minimal morphological changes were detected (Figure 1B). In the sham-operated left lung on POD 1, moderate infiltration of neutrophils and macrophages into the alveoli with focal fibrinous exudate were observed (Figure 1C). In the nonischemic opposite right lung of rats with left pulmonary IR on POD 1, moderate infiltration of macrophages into the alveoli and mild hyperemia of the alveolar septa were observed. Extensive edema and fibrinous exudates in alveolar lumina were also obvious (Figure 1D). In contrast, in the ischemic reperfused left lung of rats with pulmonary IR on POD 1, neutrophils markedly infiltrated into the alveolar septa and alveoli, and moderate infiltration of macrophages into the alveoli with focal fibrinous exudate were observed as well as severe destruction of the alveolar architecture (Figure 1E). Thereafter, severe infiltration of macrophages and increased alveolar epithelial cell proliferation were observed on POD 3 (Figure 1F), and mild infiltration of macrophages was observed on POD 5 (Figure 1G). In the reperfused left lung on POD 7, alveolar epithelial cell proliferation had decreased, and few macrophages into the alveoli were observed (Figure 1H).

Quantitative Evaluation of Lung Injury

For the quantitative analysis of lung injury, the lung injury score evaluated by histopathological examination, lung tissue MPO activity, and W/D weight ratio were examined, and the time courses of the three groups are shown in Table 1. In the sham-operated left lung, the lung injury score was slightly increased on POD 1, being about 8-fold higher than in the control left lung ($p < 0.01$). In contrast, in the ischemia-reperfused left lung of rats with pulmonary IR, the lung injury score was markedly increased and reached the peak level on POD 1, being about 20-fold higher than in the control left lung, and about 2.5-fold higher than in the sham-operated left lung ($p < 0.01$). A significant increase in lung injury score was also observed in the nonischemic opposite right lung of rats with left pulmonary IR on POD 1 compared with the sham-operated right lung (3.4 ± 0.7 versus 0.5 ± 0.3, $p < 0.01$) (data not shown). Consistent with the histopathological findings, MPO activity, a marker of tissue neutrophil infiltration, was significantly higher in the ischemic-reperfused left lung on POD 1 compared with the sham-operated left lung ($p < 0.01$). MPO activity in the sham-operated left lung was also significantly increased on POD 1 compared with the control left lung ($p < 0.05$). Lung tissue W/D ratio was significantly increased in the ischemic-reperfused left lung compared with the sham-operated left lung on POD 1 ($p < 0.01$). A significant increase in the W/D ratio was observed in the nonischemic opposite right lung of rats with left pulmonary IR on POD 1 compared with the sham-operated right lung (data not shown).

TABLE 1

TIME COURSE OF QUANTITATIVE EVALUATION OF LUNG INJURY*

	POD				
	0	1	3	5	7
Lung injury score					
Control	0	0.4 ± 0.2	0.2 ± 0.3	0	0
Sham	—	$3.2 \pm 0.8^{\dagger}$	$1.3 \pm 0.7^{\dagger}$	0.5 ± 0.4	0.3 ± 0.4
IR	—	$8.1 \pm 1.6^{\dagger,\S}$	$3.7 \pm 0.8^{\dagger,\S}$	1.3 ± 0.7	0.9 ± 0.5
MPO‖					
Control	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.2	0.4 ± 0.3	0.4 ± 0.1
Sham	—	$1.1 \pm 0.4^{\dagger}$	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.2
IR	—	$3.3 \pm 0.6^{\dagger,\S}$	0.7 ± 0.4	0.5 ± 0.2	0.5 ± 0.2
W/D ratio					
Control	4.6 ± 0.3	4.8 ± 0.6	4.6 ± 0.5	4.5 ± 0.7	4.6 ± 0.2
Sham	—	5.2 ± 0.7	4.7 ± 0.3	4.6 ± 0.5	4.6 ± 0.7
IR	—	$8.7 \pm 0.9^{\dagger,\S}$	4.9 ± 0.6	4.8 ± 0.6	4.7 ± 0.3

Definition of abbreviations: MPO = lung tissue myeloperoxidase activity; POD = postoperative day; POD 0 = before surgery; W/D ratio = wet-to-dry lung weight ratio.

* Values are mean ± SD for five rats at each time point.

† $p < 0.05$, ‡ $p < 0.01$ versus control.

§ $p < 0.01$ versus sham, Student's *t* test.

‖ MPO are expressed in absorbance changes per gram of lung tissue.

Plasma HGF Level

Blood samples were obtained from rats divided into three groups as follows: control, rats ventilated for 1 h without thoracotomy; sham, rats underwent simple left thoracotomy for 1 h; IR, rats underwent thoracotomy with left pulmonary ischemia for 1 h. The HGF concentration in plasma was measured by ELISA (Figure 2). The plasma HGF levels in the sham-operated rats gradually increased to a maximum on POD 3, and returned to the base value on POD 7. The maximum level was about 3-fold higher than that of the control rats ($p < 0.01$). In contrast, the plasma HGF levels in rats with pulmonary IR were markedly elevated on POD 1, reaching the peak level on POD 3, and then remained at high level for a week after surgery. The maximum level was about 5-fold higher than that of the sham-operated rats, and about 15-fold higher than that of the control rats ($p < 0.01$).

Tissue HGF Level in Liver and Lung

To assess the distribution of HGF, the tissue HGF concentration in the liver and lung was measured by ELISA (Figure 3). The tissue HGF levels in the liver of rats with pulmonary IR and the sham-operated rats were significantly higher than those of the control rats on POD 3 ($p < 0.05$). In the right lung, the tissue HGF levels of rats with pulmonary IR and the sham-operated rats were increased and reached the peak value on POD 3, being about 1.5-fold higher than that of the control rats ($p < 0.05$). In the left lung of the sham-operated rats, the tissue HGF level was slightly increased to 512 ± 107 ng/g tissue on POD 3. In contrast, in the left lung of rats with pulmonary IR, the tissue HGF level was markedly increased to 643 ± 161 ng/g tissue on POD 1 ($p < 0.05$) and reached the peak level of 1026 ± 185 ng/g tissue on POD 3, being about 2-fold higher than that of the sham-operated rats and about 3-fold higher than that of the control rats ($p < 0.01$). A significant increase was also observed in the ischemic-reperfused left lung on POD 5 compared with the control left lung ($p < 0.01$).

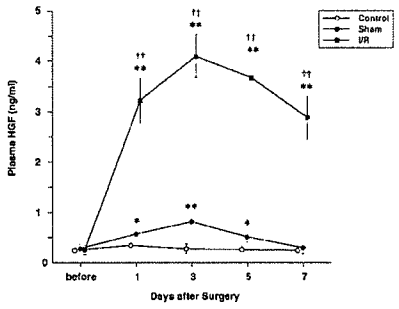

Figure 2. Time course of the plasma HGF concentration. Blood samples were obtained from rats divided into three groups as follows: control, rats ventilated for 1 h without thoracotomy (*open circles*); sham, rats underwent simple left thoracotomy for 1 h (*closed circles*); IR, rats underwent thoracotomy with left pulmonary ischemia for 1 h (*closed squares*). Values are mean ± SD for five rats in each group. *$p < 0.05$, **$p < 0.01$ versus control. ††$p < 0.01$ versus sham (Student's *t* test).

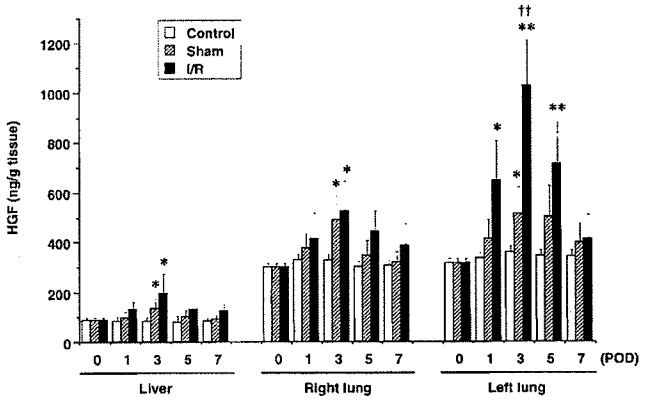

Figure 3. Time course of the tissue HGF concentration in the liver, right and left lungs. Liver and lung tissue extracts were obtained from rats divided into three groups as follows: control, rats ventilated for 1 h without thoracotomy (*open bars*); sham, rats underwent simple left thoracotomy for 1 h (*hatched bars*); IR, rats underwent thoracotomy with left pulmonary ischemia for 1 h (*closed bars*). POD = postoperative day; POD 0 = before surgery. Values are mean ± SD for five rats at each time point. *$p < 0.05$, **$p < 0.01$ versus control. $^{\dagger\dagger}p < 0.01$ versus sham (Student's *t* test).

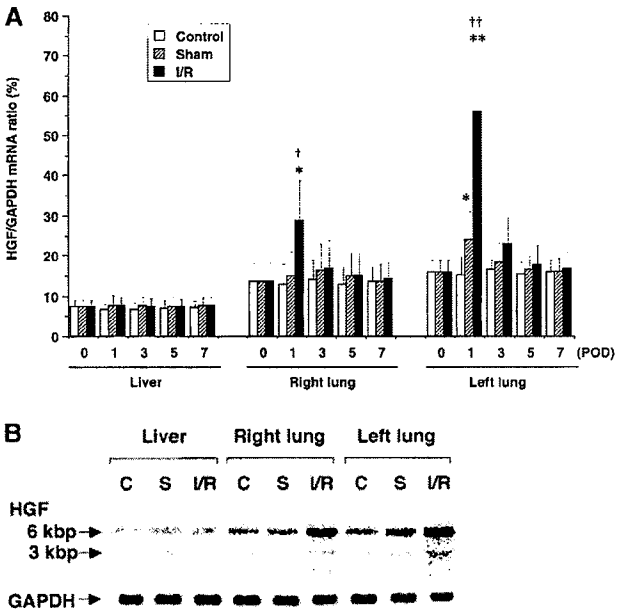

Figure 4. (*A*) Time course of the densitometric relative quantification of HGF mRNA in the liver, right and left lungs. The relative amount of HGF message was determined by scanning the bands with a Bio-Image Analyzer. For relative quantification, the densitometer value of HGF was normalized to that of GAPDH. Total RNAs were obtained from rats divided into three groups as follows: control, rats ventilated for 1 h without thoracotomy (*open bars*); sham, rats underwent simple left thoracotomy for 1 h (*hatched bars*); IR, rats underwent thoracotomy with left pulmonary ischemia for 1 h (*closed bars*). POD = postoperative day; POD 0 = before surgery. Values (%) are mean ± SD for five rats at each time point. *$p < 0.05$, **$p < 0.01$ versus control. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus sham (Student's *t* test). (*B*) HGF mRNA expression in the liver, right and left lungs. The representative results on the first postoperative day are shown. HGF mRNA levels were evaluated by Northern blot analysis, as described in METHODS. *Arrows* indicate 3 kbp and 6 kbp of rat HGF mRNA. GAPDH transcripts are shown as an internal reference for the quantification of total mRNA. C = control; S = sham; IR = pulmonary ischemia–reperfusion.

HGF mRNA Expression in Liver and Lung

To determine whether the upregulation of HGF is selective for the ischemic-reperfused lung, we examined its expression at the mRNA level by Northern blot analysis in the liver and the nonischemic right lung as well as in the ischemic-reperfused left lung of rats with pulmonary IR. The densitometric analysis of HGF normalized to the GAPDH transcripts is shown in Figure 4A. Figure 4B shows the representative results of rat HGF mRNA expression in the liver and lungs of three groups on POD 1. The level of rat HGF mRNA was significantly increased on POD 1 in the ischemic-reperfused left lung (56 ± 14%) compared with the sham-operated left lung (24 ± 7%) and the control left lung (15 ± 4%) ($p < 0.01$). Furthermore, HGF mRNA expression was also increased in the sham-operated left lung on POD 1 compared with the control left lung ($p < 0.05$), despite a similar level of HGF mRNA in the right lung between the two groups. Interestingly, a significant increase in HGF mRNA expression was also found in the nonischemic right lung of rats with pulmonary IR on POD 1 (29 ± 9%; $p < 0.05$ versus the control right lung). There were no significant differences in the HGF mRNA expression in the liver among the three groups.

Lung Immunohistochemistry

To determine the localization of HGF protein in the lung, we performed immunohistochemical analysis. The representative results of the ischemic reperfused left lung obtained from rats with pulmonary IR on POD 3 are shown in Figure 5. The cytoplasm of interstitial cells was intensely stained for HGF, but the alveolar epithelial cells were negative for HGF (Figure 5A). Most of these interstitial cells were also positive for macrophage marker (ED-1) (Figure 5B).

DNA Synthesis in the Lung after Pulmonary IR Injury

To determine the regenerative cell proliferation after pulmonary IR injury, cells undergoing DNA synthesis were identified by immunohistochemical staining using anti-PCNA monoclonal antibody. In the ischemic-reperfused left lung of rats with pulmonary IR, PCNA-positive cells were mainly alveolar epithelial cells (Figure 6). Several endothelial cells and macrophages in the alveolar space were also positive for PCNA, although the number of these cells undergoing DNA synthesis was much fewer than in alveolar epithelial cells. The PCNA labeling index for the alveolar epithelial cells was increased and reached the peak level on POD 3 in the ischemic-reperfused left lung, being about 3-fold higher than that in the sham-operated left lung ($p < 0.01$) (Table 2).

Influence of HGF Neutralization on Lung Injury and DNA Synthesis

To assess the mitogenic effect of endogenous HGF on alveolar epithelial cells of an injured lung, we injected neutralizing anti-HGF rabbit IgG (or normal rabbit IgG as a placebo) into rats with pulmonary IR and examined the influence of HGF neutralization on DNA synthesis of alveolar epithelial cells. Furthermore, we also examined its influence on lung morphology. Lung histology revealed that administration of anti-HGF IgG aggravated lung injury characterized by pulmonary edema and destruction of the alveolar architecture with few alveolar epithelial proliferation, which were more severe in rats given anti-HGF IgG than in the normal IgG-injected rats (Figure 7A). The lung injury score was significantly higher in the anti-HGF IgG-treated rats than in the normal IgG-injected rats (7.6 ± 1.4 versus 4.1 ± 1.3, $p < 0.01$) (Figure 7B). Lung immunohistochemistry revealed

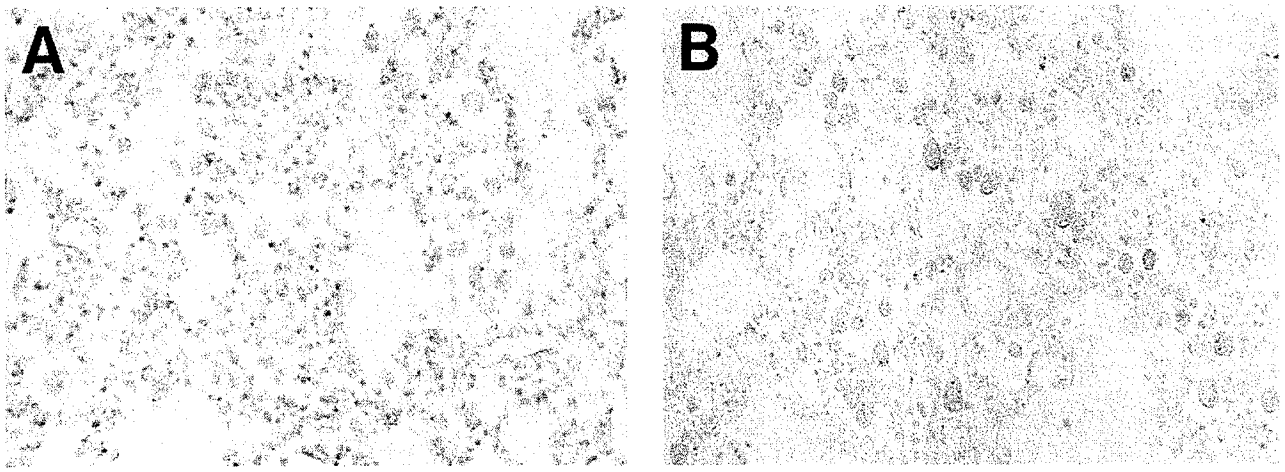

Figure 5. Immunohistochemical staining for HGF (*A*) and macrophage marker (ED-1) (*B*). The representative results of the reperfused lung obtained from rats with pulmonary IR on the third postoperative day are shown. Most of the interstitial cells that are stained for HGF are also positive for ED-1, but the alveolar epithelial cells are negative for HGF. Original magnification: ×200.

that HGF neutralization reduced the number of PCNA-positive alveolar epithelial cells (Figure 7C), and the PCNA labeling index for alveolar epithelial cells in the anti-HGF IgG-treated rats was much lower than that in the normal IgG-injected rats (3.6 ± 1.8 versus 15.3 ± 4.2, $p < 0.01$) (Figure 7D).

DISCUSSION

In the present study, we have shown that thoracotomy with 1 h of pulmonary ischemia resulted in significantly higher levels of plasma HGF compared with simple thoracotomy alone. Histopathologically, massive neutrophil infiltration in interstitium and alveoli was observed as well as destruction of the alveolar architecture in the ischemic-reperfused lung. Furthermore, lung tissue MPO activity, a marker of tissue neutrophil infiltration, was also augmented in the reperfused lung. Sekido and colleagues (29) have provided evidence that ablation of neutrophils attenuated the degree of tissue injury in rabbit models of pulmonary IR injury, indicating a pivotal role of neutrophils in the pathogenesis of IR injury. Therefore, our results suggest that HGF production may be associated with lung injury caused by pulmonary IR in our rat model, which was established by occlusion of the left lung. In our pilot study, thoracotomy with 1 h of pulmonary compression, in which the left lung was compressed using a balloon filled with water, resulted in significantly higher levels of plasma HGF compared with simple thoracotomy, but significantly lower HGF levels compared with thoracotomy with pulmonary ischemia. We also measured the volumetric lung tissue blood flow in the compressed lung using a noncontact laser doppler blood flowmeter, and those values rapidly decreased to 30% of the normal values during simple thoracotomy (data not shown). These findings suggest that the plasma HGF level reflects the severity of acute lung injury caused by hypoxia and subsequent reoxygenation.

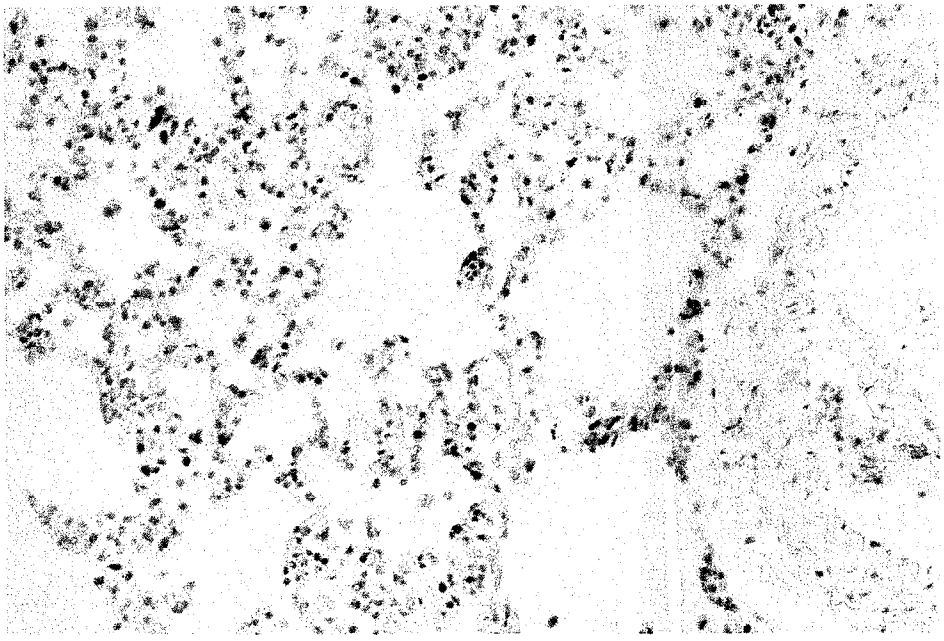

Figure 6. Distribution of cells undergoing DNA synthesis in the ischemic-reperfused left lung. DNA synthesis was identified by immunohistochemical staining using anti-PCNA monoclonal antibody, and the representative result obtained from rats with pulmonary IR on the third postoperative day is shown. Dark brown nuclei represent positive staining. PCNA-positive cells were mainly alveolar epithelial cells. Several endothelial cells and macrophages in the alveolar space were also positive for PCNA, although the number of these cells undergoing DNA synthesis was much fewer than in alveolar epithelial cells. Original magnification: ×200.

TABLE 2

PCNA LABELING INDEX FOR ALVEOLAR EPITHELIAL CELLS*

	POD				
	0	1	3	5	7
Control	0.6 ± 0.2	0.9 ± 0.4	0.7 ± 0.3	0.7 ± 0.4	0.6 ± 0.3
Sham	—	2.7 ± 1.3†	6.1 ± 2.5‡	1.1 ± 0.9	0.7 ± 0.5
IR	—	4.9 ± 2.1†	19.7 ± 5.8‡,‖	11.7 ± 5.7§	3.6 ± 2.3

Definition of abbreviations: POD = postoperative day; POD 0 = before surgery.
* Values are mean ± SD for five rats at each time point.
† $p < 0.05$, ‡ $p < 0.01$ versus control.
§ $p < 0.05$, ‖ $p < 0.01$ versus sham, Student's *t* test.

Northern blot analysis revealed that HGF mRNA was dramatically upregulated in the reperfused lung after pulmonary ischemia. However, HGF mRNA was upregulated even in the nonischemic left lung of rats with simple left thoracotomy, suggesting that HGF expression may be induced by surgical trauma such as thoracotomy alone, unrelated to pulmonary IR injury. Nevertheless, the HGF mRNA level was significantly higher in the ischemic-reperfused left lung compared with the nonischemic left lung of rats with simple left thoracotomy, indicating that enhanced expression of HGF mRNA is associated with pulmonary IR injury. Two sequence elements, an interleukin-6 (IL-6) response element and a potential binding site for nuclear factor IL-6, are located near the transcription initiation site of the human HGF gene, and they might be involved in the regulation of HGF gene expression (30). It has been also reported that inflammatory cytokines such as IL-1β and tumor necrosis factor-α stimulate HGF production (31). Recently, we reported that serum IL-6 levels are markedly increased after thoracic surgery (23), and IL-1β gene expression is upregulated in pulmonary IR injury (32). These findings suggest that pulmonary IR injury and surgical trauma may induce the production of these inflammatory cytokines, which in turn enhance the upregulation of HGF gene expression in an injured lung.

In this study, HGF mRNA was also upregulated even in the nonischemic opposite right lung after left pulmonary ischemia, although the mRNA level was much higher in the injured left lung compared with the intact right lung. Moreover, no significant increase in HGF mRNA level was found in the right lung of the sham-operated rats. These results suggest that HGF mRNA in the intact right lung after left pulmonary ischemia may be regulated by factors other than pure pulmonary IR injury. The relative increase in blood flow to the opposite right lung during occlusion of the left lung may be in part responsible for the upregulation of HGF mRNA in the intact right lung. Indeed, the histological examination revealed mild hyperemia of the alveolar septa and severe intraalveolar edema in the opposite right lung of rats with left pulmonary IR. However, further investigations are needed to explain the expression of HGF in the nonischemic right lung.

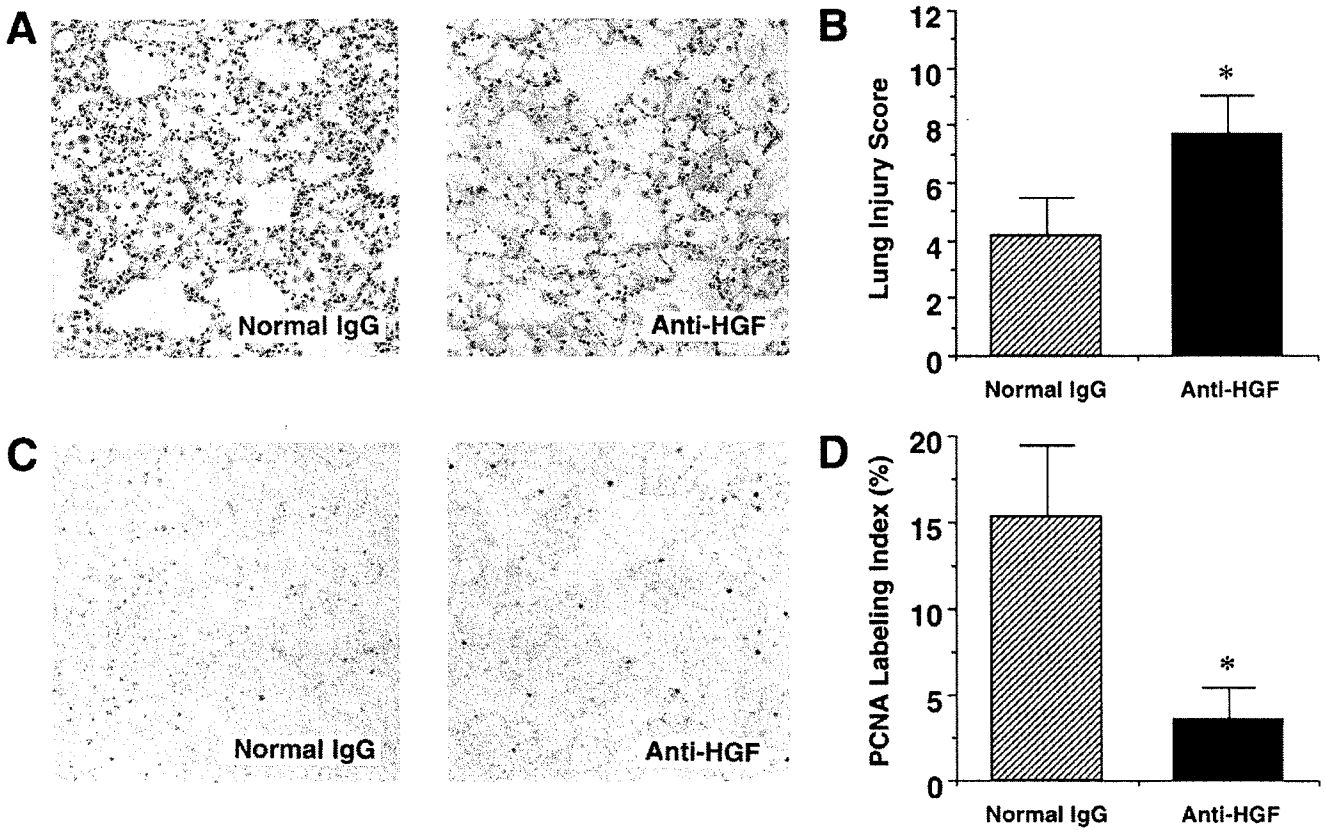

Figure 7. Influence of HGF-neutralizing treatment with an anti-HGF antibody on lung injury and DNA synthesis of alveolar epithelial cells. (*A*) Histological findings of the ischemic-reperfused lung in rats given normal or anti-HGF IgG (hematoxylin–eosin stain; original magnification: ×100), and (*B*) the quantitative analysis of lung injury, as determined by lung injury score. (*C*) Immunohistochemical staining for PCNA in the ischemic-reperfused lung of rats given normal or anti-HGF IgG (original magnification: ×100), and (*D*) the quantitative analysis of DNA synthesis, using PCNA labeling index for alveolar epithelial cells. Values are mean ± SD for six rats in each group. *$p < 0.01$ compared with the value in normal IgG-injected group (Student's *t* test).

HGF is secreted as a single-chain precursor. In response to tissue injury, the single-chain precursor is converted to a biologically active heterodimer by a serine protease, the activity of which is initiated in the injured tissue (33). We found here that the tissue HGF levels of the sham-operated rats were significantly higher not only in the left lung but also in liver and right lung compared with those of the control rats, despite similar levels of HGF mRNA in the liver and right lung between the two groups. The discrepancy in the degree of increase in HGF levels between HGF mRNA and the tissue HGF may be due to the difference in measurement. HGF mRNA correlates to the full-length single-chain HGF, whereas the antibody used for measurement of tissue HGF level was raised against a peptide corresponding to the α-chain of HGF, and thus recognizes the intracellular monomeric forms as well as the mature heterodimeric forms (24). Furthermore, HGF has a specific affinity for heparin, and there are the high- and low-affinity HGF receptors present on the target cell surface (34). The high-affinity receptor, which corresponds to a signal-transducing receptor, is the c-*met*/HGF receptor, whereas the low-affinity receptor is thought to be mainly heparin sulfate-like polysulfated glycosaminoglycan. Thus, the tissue HGF level may include not only the cytoplasmic HGF but also the receptor-bound HGF and the membrane-bound HGF captured and retained by proteoglycans present on the extracellular matrix and cell surface, although the blood was removed thoroughly from the organs by saline perfusion. These variant forms of HGF protein and the receptor-bound HGF could explain the disparity between the elevated protein values in sham liver and right lung where mRNA did not increase.

In this model, pulmonary IR caused neutrophil infiltration and subsequently increased cellularity with alveolar macrophages and alveolar epithelial cell proliferation. Furthermore, DNA synthesis of alveolar epithelial cells was significantly increased in the ischemic-reperfused lung. In the injured lung, the bronchial and alveolar type II epithelium replications are essential for tissue repair and regeneration (14). Recent studies have demonstrated that HGF stimulates proliferation of these epithelial cells through DNA synthesis *in vitro* and *in vivo* (15–19). Taking these results together, it is tempting to speculate that pulmonary IR caused lung injury, which in turn enhanced the expression of HGF for regeneration of an injured lung. To examine our hypothesis, we administered neutralizing anti-HGF antibody into rats with pulmonary IR. HGF neutralization dramatically inhibited DNA synthesis of alveolar epithelial cells in the ischemic-reperfused lung. Furthermore, anti-HGF treatment aggravated lung injury, whereas the control antibody did not. These results suggest that HGF may act as a pulmotrophic factor for regeneration of an injured lung after pulmonary IR. In the lung, the major sources of HGF are mesenchymal cells, such as alveolar macrophages, fibroblasts, and endothelial cells (15, 21, 35). We found here the alveolar macrophages were intensely stained for HGF. These findings suggest that HGF produced by the alveolar macrophages may induce proliferation of the alveolar epithelial cells in the injured lung.

This experimental study supports and extends our recent work, which has demonstrated that the serum HGF level is markedly elevated during the early period after transthoracic esophagectomy (23). We have clinically noticed that pulmonary infections are frequent after transthoracic esophagectomy and some cases progressed to severe respiratory failure. Taking our results together, severe surgical trauma such as pulmonary ischemia during thoracic surgery may cause lung injury, which facilitated pulmonary infections through destruction of the alveolar architecture that acts as a physiological barrier. In light of this information, prevention or early treatment of lung injury after thoracic surgery may reduce the frequency of postoperative pulmonary infections. Recent studies have shown a therapeutic potential of HGF in acute lung injury and bleomycin-induced lung fibrosis (36, 37). The present study suggests that HGF may act as a pulmotrophic factor for lung regeneration after acute lung injury caused by pulmonary IR. In this context, exogenous HGF may be a potential candidate as a preventive agent for pulmonary infections after major thoracic surgery. Furthermore, even in the established postoperative pulmonary infections, exogenous HGF may also have a therapeutic potential through regeneration of an injured lung.

In conclusion, we have shown that enhanced expression of HGF and DNA synthesis of alveolar epithelial cells were induced in the ischemic-reperfused lung. Furthermore, HGF-neutralizing treatment with an anti-HGF antibody dramatically reduced DNA synthesis of alveolar epithelial cells in the reperfused lung and aggravated lung injury. These findings suggest that HGF may play an important role in regeneration of an injured lung after pulmonary IR. This study may provide an important consideration in understanding the basic biological process of lung regeneration after pulmonary IR injury. In addition, this work may contribute to new preventive and therapeutic strategies for lung injury after major thoracic surgery, such as transthoracic esophagectomy and lung transplantation, accompanied by a prolonged pulmonary ischemia. Further clarification of the biological and physiological significance of HGF in pulmonary IR will likely have important clinical implications in light of "regenerating medicine" in the future.

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Relationship of Keratinocyte Growth Factor and Hepatocyte Growth Factor Levels in Rat Lung Lavage Fluid to Epithelial Cell Regeneration after Bleomycin

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Keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) are known mitogens for normal alveolar Type 2 cells *in vitro* and *in vivo*. We wished to determine whether these two growth factors are involved in lung repair after epithelial cell necrosis by determining the levels of each factor in lung lavage fluid collected serially after bleomycin-induced injury, and how these values relate specifically to proliferation of bronchiolar and alveolar epithelial cells. Rats received an intratracheal injection of 1 unit bleomycin in 0.5 ml water and were killed at intervals up to 4 weeks with 1 μCi/g tritiated thymidine injected 1 hour before death. Early necrosis of bronchiolar epithelial (BR) cells and Type 1 alveolar epithelium was followed by an increase in inflammatory cell numbers and high protein levels in bronchoalveolar lavage (BAL) fluids. In addition, the levels of KGF and HGF, measured by enzyme-linked immunosorbent assay in BAL, increased as early as 3 days and peaked at 7–14 days, when KGF was measured at 160 pg/ml (n = 50) and HGF reached 460 pg/ml (n = 40). Both values dropped sharply after 2 weeks. Epithelial cell proliferation was quantitated as percentage of labeled cells in autoradiographs of methacrylate sections. Labeling of BR cells predominated in the first week and peaked at 7% at 3 days. Type 2 cell proliferation was delayed somewhat but occurred in 3 to 10 days with a peak of 7% labeled cells at 1 week. The results demonstrate that both HGF and KGF are present in the lung in greatly increased amounts soon after bleomycin-induced epithelial cell necrosis. These high levels are associated with both BR and alveolar epithelial cell proliferation. *(Am J Pathol 1999, 155:949–954)*

Widespread necrosis of alveolar and bronchiolar epithelial cells in the lung is often followed by a delayed, sometimes abnormal epithelial regenerative pattern while the fibrotic process predominates. This type of response is seen clinically in examples of fibrosing alveolitis and in various experimental models of this disease.[1–3] Although many studies concentrate on the role of macrophage activation and its cytokine secretions in the evolution of pulmonary fibrosis, there is also evidence that the regulation of epithelial cell regeneration is important in the pathogenesis. Rapid epithelial proliferation can restore the surface layer and prevent fibroblast migration to the air spaces and is associated with control of underlying fibroblasts.[3,4]

Identification of molecules that induce pulmonary epithelial cell proliferation is therefore important. Several recent studies have indicated that two cytokines have this potential, namely hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF), also known as fibroblast growth factor-7. These molecules stimulate normal alveolar Type 2 cell proliferation *in vivo* and *in vitro*.[5–7] They also appear to promote bronchiolar cell proliferation[7,8] and have been shown to be essential for normal lung development.[9] The role of KGF and HGF in repair is less certain; for example, when KGF is administered after lung injury induced by bleomycin, there is no change in outcome and no accelerated repair.[10] However, if KGF is given before injury is induced, a protective effect is seen; this has been shown in several models of lung injury.[10–12] This leads to the question of what role HGF and KGF play *in vivo* in stimulating Type 2 cells and bronchiolar epithelial cells to proliferate after injury. We studied this relationship by using intratracheal bleomycin in rats to induce injury to these two pulmonary epithelial cell populations. Subsequently, we measured levels of KGF and HGF in lung lavage fluids at several time points from initial injury to fibrotic repair and correlated levels of these factors specifically with the proliferative responses of both bronchiolar and alveolar epithelial cell populations.

Materials and Methods

Male Sprague-Dawley rats weighing 180 to 200 g were injected intratracheally (i.t.), while under mild barbiturate-

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induced anesthesia, with 5 U/kg bleomycin (Bristol-Myers Squibb Co., Evansville, IN) in 0.5 ml sterile water. Animals were killed by intraperitoneal (i.p.) barbiturate overdose in groups of 4 at 0, 3, 7, 10, 14, and 28 days after i.t. bleomycin. Each rat received an i.p. injection of tritiated thymidine (^{3}HT) at 1 μm Ci/g 1 hour before death. Two control animals per group received 0.5 ml water only (i.t.) and were killed at the same time intervals.

On each animal, the chest was opened and the lungs allowed to collapse. A tracheotomy was performed and the lungs lavaged twice with 5 ml of Tris-based saline solution containing 10 μl per ml of protease inhibitor cocktail (Sigma, St. Louis, MO). After lavage, the 2 samples per animal, about 9 ml, were pooled then 5 μl of 20% bovine serum albumin and 5 μl of Tween ~20 were added per milliliter of fluid. An aliquot was taken to count cells by hemocytometer and the remainder was centrifuged to separate the fluid component, which was divided into several aliquots and frozen at −70°C. One aliquot was used later to measure protein content[13] and the remainder to measure growth factors by enzyme-linked immunosorbent assay (ELISA).

Lung Tissue

After lavage, the left lobes of lung were tied off, removed, and frozen for later measurement of total hydroxyproline.[14] The right lobes were re-inflated with buffered formaldehyde overnight, then 6 random tissue blocks approximately 5 × 5 × 8 mm were cut for processing and embedded in glycol methacrylate. Three random blocks per rat were sectioned at a thickness of 0.75 μm and used for autoradiography.[15] On each of the 3 sections, the overall percentage of ^{3}HT-labeled cells was determined after counting 1500 cells per slide. The initial field was set at the pleural surface about midlobe and the field moved systematically back and forth across the lobe until 1500 cells were recorded. The percentages of labeled cells per animal and per group at each time were then calculated. Subsequently, using the same sections, 1000 bronchial and bronchiolar (BR) epithelial cells were identified and the number labeled was recorded. Also, 1000 alveolar Type 2 epithelial cells were identified by their shape, location and lamellar body contents; the number incorporating ^{3}HT was recorded. To obtain enough BR and Type 2 cells, usually the whole sections had to be counted and the numbers then used to calculate the percentages of labeled BR and alveolar epithelial cells at each time interval after administration of bleomycin. In lavaged lungs, any remaining alveolar macrophages were usually round and free from the alveolar wall. These cells were very rarely labeled.

ELISA

The bronchoalveolar lavage (BAL) fluid was used to measure KGF and HGF using standard ELISA methods. Samples of 100 μl of BAL were used for duplicate plate readings at each time interval per animal per group. The antibodies used and the growth factor standards were obtained from R & D Systems (Minneapolis, MN). These agents were designed specifically to measure human growth factors, and we obtained reproducible linear standard values using ELISA on human HGF and KGF diluted in our BAL solution. A protein in the rat lung BAL fluids also bound to each human antibody during a 2-hour incubation period and the ELISA showed linear results on dilution. Measurements were expressed as pg/ml BAL and both assays were sensitive to a level of 5 pg/ml.

In a subsequent test, we obtained an ELISA kit for rat HGF (Institute of Immunology, Tokyo). We ran one set of the above BAL fluids and the growth factor controls with 22-hour incubation at room temperature using instructions provided by the manufacturer. The level of rat HGF was quantitated by ELISA. Samples of lavage fluid were also incubated with antibody to rat HGF to test whether it neutralized the factor measured by ELISA.

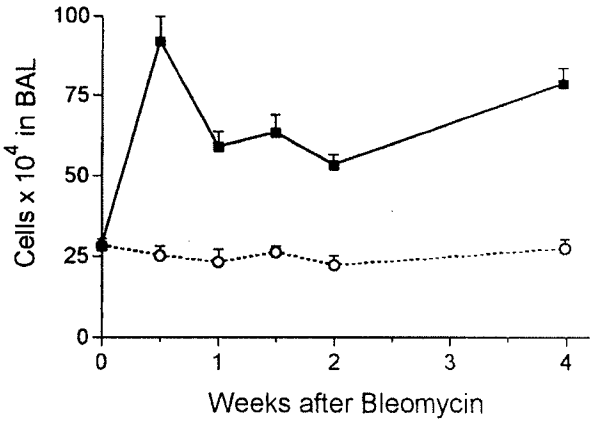

Figure 1. Total number (mean ± SE) of cells recovered by BAL at intervals after bleomycin (solid squares) or water (open circles). All experimental groups > control ($P < 0.05$) at same time point.

Results

Lung Injury

Bleomycin induced immediate lung injury and an inflammatory response as previously described.[2,4] Epithelial cell necrosis at the bronchiolar and alveolar levels was seen and many inflammatory cells were observed nearby. The total number of these cells recovered by BAL was increased threefold at 3 days and continued at about double the normal value for the 4-week period (Figure 1). The cytospin preparations showed these cells to be mixed alveolar macrophages and polymorphonuclear leukocytes. Further evidence of injury was found in the levels of protein in the BAL fluid. Control levels were always in the 80–100 μg/ml range, but 3 days after bleomycin, the alveolar protein level peaked at 8 times this value (Figure 2). The protein level then fell steadily but did not reach the base value by the end of the 4-week experimental period. The amount of hydroxyproline measured in lung tissue rose steadily after 1 week and was more than twice the normal amount by 4 weeks (Figure

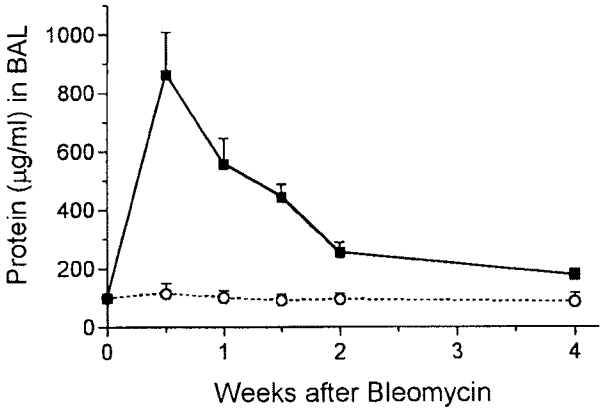

Figure 2. Protein content in μg per ml BAL fluid after instillation of bleomycin (solid squares) or water (open circles). All experimental groups > control ($P < 0.05$) at same time.

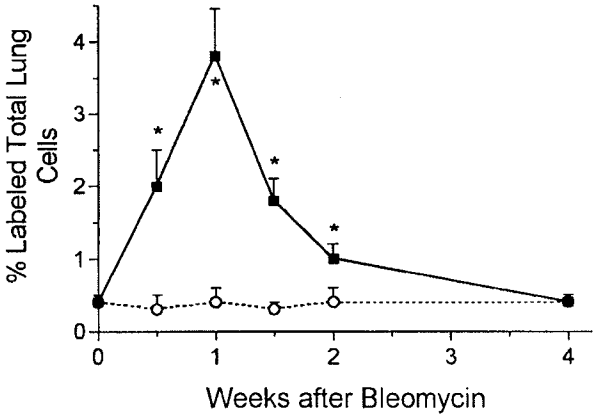

Figure 4. Percentages of total lung cells that incorporated ^{3}H thymidine at intervals after bleomycin (solid squares) or water (open circles). *, $P < 0.05$ > control.

3). From 2 to 4 weeks, evidence of pulmonary fibrosis was seen readily by light microscopy.

Autoradiography

The percentage of ^{3}HT-labeled lung cells in noninjected rats and in all of the water-injected controls was always around 0.3–0.4%. In bleomycin-injected rats, counting all cell types in the lung, the overall percentage of labeled cells rose to about 2% at day 3, then doubled again, peaking at 1 week (Figure 4). The level then fell but did not return to the control range until 4 weeks. The vast majority of labeled cells in the first 10 days after bleomycin was administered were epithelial cells. Labeled BR epithelium was particularly prominent at 3 and 7 days, whereas Type 2 alveolar epithelium was most prominently labeled at 7 and 10 days (Figures 5 and 6). When these epithelial types were identified and counted specifically at each time point, it was found that BR cells showed maximal labeling at 3 days and that labeling returned to normal by 2 weeks, whereas the rise in Type 2 cell labeling was somewhat slower and peaked at 7% of cells at 1 week before declining by 2 weeks (Figure 7).

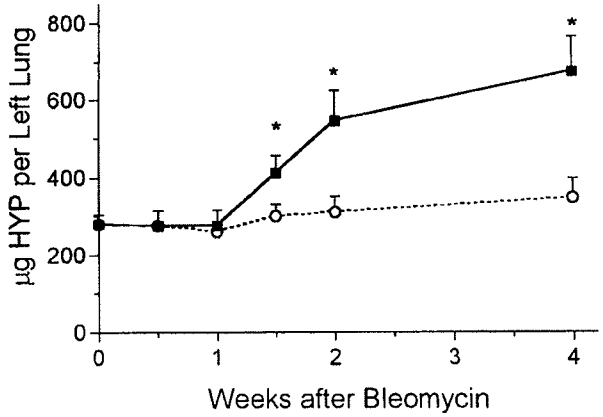

Figure 3. Total hydroxyproline content (μg) per left lobe of lung after bleomycin (solid squares) or water (open circles). *, $P < 0.05$ > control.

Most of the ^{3}HT labeling at 2 weeks and beyond was found in interstitial fibroblasts.

Growth Factors in BAL

From each animal approximately 9 ml of fluid was recovered from the double lavage procedure. In the control rats, about 50 pg/ml of KGF was measured and the value was unchanged after water instillation. Bleomycin injection resulted in a rapid increase in KGF levels in lavage

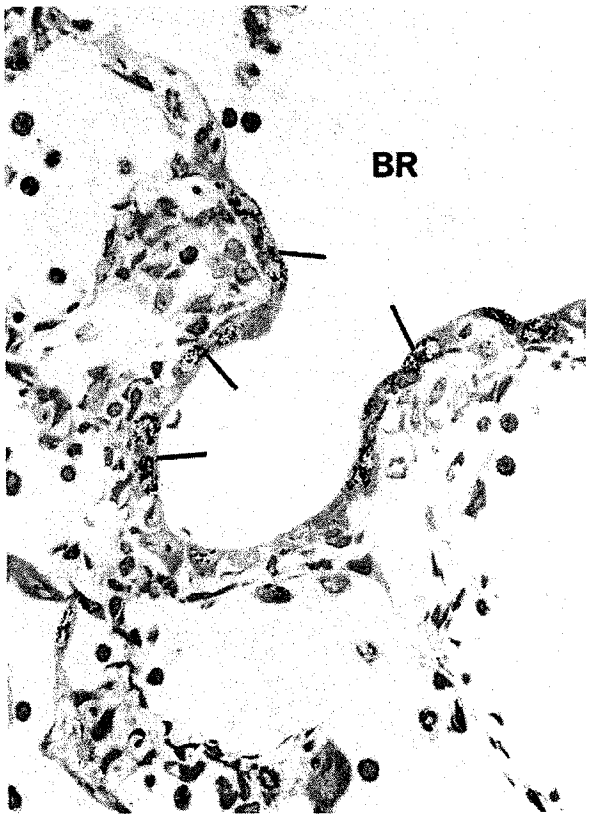

Figure 5. Autoradiograph of lung 3 days after bleomycin shows many labeled epithelial cells (arrows) of the bronchiolar epithelium (BR) × 950.

Figure 6. Autoradiograph of an alveolar (ALV) region of lung 7 days after bleomycin shows ^{3}H thymidine-labeled Type 2 cells (arrows) × 950.

fluid, with peak levels occurring between 1 and 2 weeks at around 150 pg/ml (Figure 8). The value returned to the normal range by 4 weeks. A much greater increase in HGF was induced by bleomycin treatment. The level rose from control value of 40 pg/ml to about 200 pg/ml at day 3 before peaking at 10 times normal at day 10 (Figure 9). The HGF level had returned to normal by 4 weeks.

These results were obtained using the kit for human HGF. To confirm that we were measuring HGF in the rat lung, we subsequently ran these samples using a specific rat HGF ELISA kit. The values obtained and the shape of the graph were similar to that shown in Figure 9 (data not shown). Control levels of HGF were around 70

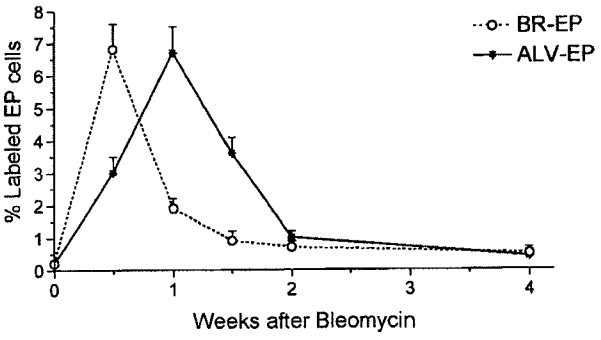

Figure 7. Percentages of bronchiolar epithelial cells (BR-EP) and alveolar epithelial cells (ALV-EP) labeled by ^{3}H thymidine after bleomycin. Control values were always the same as for time 0.

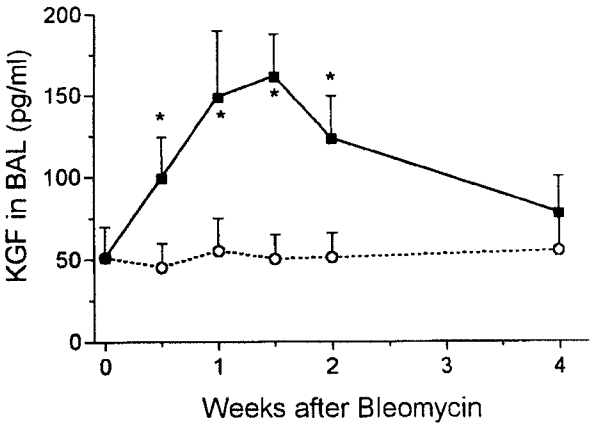

Figure 8. Levels of KGF in BAL fluid at intervals after bleomycin (solid squares) and water (open circles). *, $P < 0.05$ > control.

pg/ml BAL and rose after bleomycin to peak at 400 pg/ml on day 10. In addition, the HGF measured in human assay system was almost all neutralized when BAL samples were first incubated with the anti-rat HGF.

Discussion

The intratracheal instillation of bleomycin is a widely used model for the rapid induction of fibrosing alveolitis in experimental animals. The initial events involve necrosis of bronchiolar and alveolar Type 1 cells, and abnormal epithelial repair is a feature of the subsequent pathological changes that lead to pulmonary fibrosis.[1–4] These same features were observed in this study, in which the early epithelial cell injury was followed by an inflammatory response and the leakage of protein to the air spaces. The acute injury phase lasted 2 weeks, as indicated by the elevated protein levels in BAL fluid, and subsequently fibrosis could be seen in sections and measured biochemically. These results confirm the validity of this model system in the rat for the study of lung injury and repair.

Increased cell proliferation in the lung began quite soon after injury was induced. The autoradiographs indi-

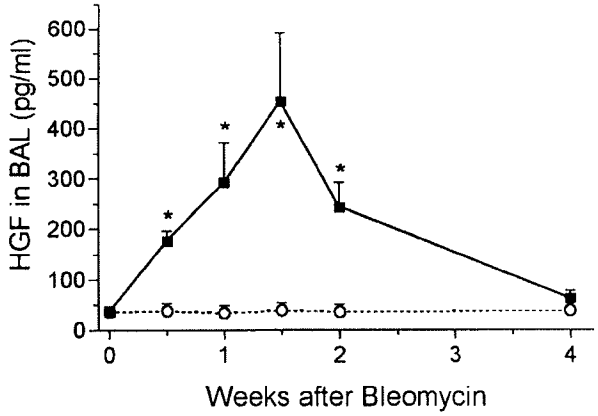

Figure 9. Levels of HGF in BAL fluid at intervals after bleomycin (solid squares) and water (open circles). *, $P < 0.05$ > control.

cated that DNA synthesis was 4 to 8 times normal over days 3 to 10 after administration of bleomycin, at which time the bulk of labeling was over the epithelial cell populations in the distal lung. It is well known that bleomycin is toxic to bronchiolar epithelial cells and Type 1 alveolar epithelium; several studies, including work from our laboratory, have reported altered Type 2 cell kinetics and differentiation after bleomycin.[2,4] In the present study, we concentrated specifically on the epithelial populations, independently identifying and counting BR and alveolar epithelial cells. The results demonstrate that, although injury occurs simultaneously in these cell populations at day 0, their regenerative patterns are not in step with one another. The BR epithelial cells show a very rapid repair pattern in which labeling peaks at 3 days and proliferation is near normal again by 10 days, whereas DNA synthesis by Type 2 cells increases more slowly, with a peak at 1 week and a return to normal by 2 weeks. The more rapid repair of the BR epithelium may explain the return to nearly normal morphology at this anatomical site, whereas the somewhat slower repair at the alveolar surface is associated with subsequent abnormal epithelial differentiation.[2,4]

In a situation analogous to wound repair, rapid restoration of a normal pulmonary epithelium after acute injury is associated with an absence of fibrous scarring.[3,4] Factors that can promote accelerated repair have been sought and recent studies have shown that KGF can induce Type 2 cell division in culture and also when instilled to normal lung.[5,6] This mitogen is produced and secreted by fibroblasts, then binds exclusively to epithelial cells,[5,16] affecting various organs with a high epithelial cell component, including the lung. Most studies of KGF effects on lung cells have been *in vitro* or have involved injecting KGF in attempts to accelerate repair. In the latter cases, KGF has been found to be more beneficial when given before injury, implying a protective action on the alveolar epithelium, possibly by acting as an antioxidant.[10–12] There is little evidence of endogenous KGF production in the lung during injury or of how levels of this growth factor relate to the regeneration of various types of epithelium. In this report we show evidence that KGF is secreted rapidly after injury and that it can be detected in BAL fluid. After bleomycin-induced acute lung injury, the KGF level rose threefold, suggesting that the fibroblast production is up-regulated. This increase in KGF coincided with an increase in BR and alveolar epithelial cell proliferation, whereas higher than normal levels of KGF persisted even after the epithelial growth phase was over. Epithelial proliferation may have slowed as contact inhibition was reestablished, even though cytokine levels remained elevated.

A similar pattern of growth factor activity was seen when HGF was measured. Levels of this molecule in BAL were much higher and reached about 10 times control values within a few days after bleomycin-induced injury, then also stayed above normal when the phase of increased epithelial DNA synthesis was over. HGF has also been shown to be a mitogen for pulmonary epithelial cells[5,9] and is synthesized by mesenchymal cells, which include lung fibroblasts and endothelial cells.[7,17] As with KGF, there is little precise evidence to relate HGF's presence in the lung with epithelial cell proliferation during repair because, in cases of lung injury where HGF has been measured, the proliferative status of the epithelium has not been clear.[18] In that study of patients with acute lung injury, levels of both HGF and KGF were found to be significantly raised in edema fluid, with HGF reaching much higher levels. In addition, the concentration of HGF in those patients was 7 times higher in edema fluid than in plasma, indicating a pulmonary origin for this growth factor.[18] The results of the present study show that two potent mitogens, KGF and HGF, are secreted and are present at high levels in the lung soon after epithelial cell necrosis. Although most attention to KGF and HGF has been directed at effects on epithelial cell proliferation, there is evidence that these factors may affect other properties of the reparative process. For example, KGF promotes cell migration and spreading of Type 2 cells,[19] which is essential if the regenerating epithelium is to cover the alveolar surface. In addition, KGF up-regulates the production of surfactant proteins in cultured cells[20] and may enhance alveolar fluid clearance after injury by up-regulating transepithelial ion transport.[21] In addition, these two growth factors may work in concert, as the administration of both at the same time further accelerates epithelial cell proliferation.[9]

The present study shows that both HGF and KGF are present at high levels in the lung soon after injury at a time when epithelial cell proliferation occurs. Although most of our ELISA results were obtained using a detection system for human growth factors, there is >90% homology between the human and rat molecules, and we were able to measure a similarly increased level of HGF using a kit specific for rat HGF. We have not directly demonstrated a causal role for these cytokines in epithelial repair, but the fact that these two well known epithelial mitogens are up-regulated soon after injury is strong evidence of a causal relationship. There are few other known mitogens for Type 2 cells and, in an earlier study, it was shown that most epithelial proliferative activity in rat lung lavage fluid was due to HGF.[22] The fact that the level of protein in BAL fluid was maximal at 3 days, then fell sharply, while the levels of KGF and HGF continued to increase to their maximum levels at 1 to 2 weeks, indicates that these cytokines did not arise from leaked plasma proteins during early edema, but were more likely to have been produced in the lung.

The rapid production and secretion of epithelial growth factors soon after injury suggests that any delay in the epithelial proliferative response may be due to a failure to up-regulate receptors rather than to a deficiency of growth factor. The presence of high levels of endogenous growth factors may explain why the administration of additional growth factors does not accelerate repair.[10,12] Our results suggest that there may be enough HGF and KGF present so that any additional injections would be superfluous, although some studies show that growth factor injection before injury has some benefit, possibly due to DNA repair or to an unknown protective mechanism.[10–12] It appears, however, that acute injury such as that induced by bleomycin, although damaging to epi-

thelial cells, does not inhibit the fibroblast from producing KGF, for example. Bleomycin administration results in the rapid induction of tumor necrosis factor-α,[23] which in turn can cause the up-regulation of KGF production by fibroblasts.[16] This suggests that after epithelial cell injury, when high levels of epithelial growth factors are present in the lung, any delay in epithelial cell proliferation may be due to the extent of damage, possibly including injury to progenitor cells such as the Type 2 epithelium. Such injury could prevent the up-regulation of receptors for HGF and/or KGF on these cells, thereby inhibiting their regenerative response.

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Hepatocyte Growth Factor and Keratinocyte Growth Factor in the Pulmonary Edema Fluid of Patients with Acute Lung Injury

Biologic and Clinical Significance

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Hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) are among the most potent mitogens identified for alveolar type II epithelial cells and may have other important functions in repair of the alveolar epithelium in acute lung injury (ALI). However, neither growth factor has been identified in the distal air spaces or plasma of patients with ALI. The goals of this study were to determine: (*1*) whether HGF and KGF are present in pulmonary edema fluid from patients with ALI and control patients with hydrostatic pulmonary edema; (*2*) whether HGF and KGF are biologically active in pulmonary edema; and (*3*) whether HGF or KGF levels are associated with clinical outcome. Pulmonary edema and plasma samples were obtained within 48 h of onset of acute pulmonary edema requiring mechanical ventilation in 26 patients with ALI and 11 control patients with hydrostatic edema. HGF and KGF concentrations were measured with enzyme-linked immunosorbent assays (ELISAs). The median (25th to 75th percentiles) concentration of HGF in pulmonary edema fluid was 21.4 (8.3 to 41.3) ng/ml in ALI and 6.6 (4.8 to 11.4) ng/ml in hydrostatic edema fluid ($p < 0.01$). The HGF concentration was 7-fold higher in the edema fluid than in the plasma of patients with ALI. In contrast, KGF was detected in low concentrations in edema fluid of patients with ALI and hydrostatic pulmonary edema; the concentration of KGF did not differ in ALI edema (0.6 [0.3 to 2.1] ng/ml) and hydrostatic edema fluid (0.2 [0.0 to 2.6] ng/ml) (p = NS). HGF and KGF were partly purified from four edema-fluid samples by heparin–Sepharose chromatography. Partly purified edema fluids were potent stimuli of DNA synthesis in cultured rat type II alveolar cells; addition of neutralizing antibodies to HGF and KGF attenuated this increase in DNA synthesis by 66% and 53%, respectively. Interestingly, higher edema-fluid levels of HGF were associated with higher mortality in patients with ALI. These novel results show that HGF and KGF are active in the alveolar space early in ALI, probably mediating early events in lung repair, and that increased levels of HGF in edema fluid may have prognostic value early in ALI. **Verghese GM, McCormick-Shannon K, Mason RJ, Matthay MA. Hepatocyte growth factor and keratinocyte growth factor in the pulmonary edema fluid of patients with acute lung injury: biologic and clinical significance.

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Several studies have demonstrated morphologic injury to the alveolar epithelial barrier in patients with clinical acute lung injury (ALI) (1–3). In addition, there is evidence of impaired surfactant release and altered alveolar epithelial-fluid transport in many patients with ALI (4–6). The early phase of recovery from ALI requires proliferation and migration of alveolar type II cells in order to restore the integrity of the denuded alveolar epithelium (7). Because hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) have recently been discovered to be the principal mitogens for alveolar type II cells, these growth factors may contribute to alveolar epithelial repair following lung injury. HGF is a unique, heparin-binding, proteolytically activated heterodimer produced primarily by endothelial and mesenchymal cells of the lung, liver, kidney, and adrenal gland (8–11). KGF is the seventh member of the fibroblast growth factor (FGF) family, secreted only by fibroblasts, and mitogenic specifically to epithelial cells (12–14). Both HGF and KGF are important mediators of interactions between the epithelium and mesenchyme following tissue injury, and are important cell motogens that can regulate wound closure (15–18).

Since establishment of the importance of HGF in experimental models of injury, several studies have reported in-

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creased plasma levels of HGF in patients with fulminant hepatitis, acute pancreatitis, hypertension, and inflammatory lung diseases (19–23). Furthermore, higher serum concentrations of HGF correlated with greater severity of illness, and decreasing serum levels of HGF were associated with clinical improvement in patients with interstitial pneumonitis and bacterial pneumonia (20, 22). However, HGF has not been measured in the plasma and alveolar edema fluid of patients with ALI. KGF has not yet been identified in the serum or alveolar fluid of patients with ALI, although several experimental studies have already shown that intratracheal administration of KGF prior to induction of lung injury with hyperoxia, radiation, bleomycin, acid-aspiration, or α-naphthylthiourea attenuates the severity of lung injury (24–28).

On the basis of the experimental data, HGF and KGF may be important in mediating recovery from clinical ALI, and might also have prognostic and even therapeutic value for the care of patients with ALI. However, neither growth factor has yet been identified in the edema fluid or plasma of patients with ALI. Therefore, the first objective of the present study was to determine whether HGF and KGF are present in biologically significant quantities in pulmonary edema fluid and plasma of patients with ALI, and if so, to determine whether HGF and KGF are biologically active. In order to control for the effect of pulmonary edema resulting in the need for positive-pressure ventilation (29), the second objective was to determine whether HGF and KGF concentrations are higher in patients with ALI than in control patients with hydrostatic pulmonary edema. The third objective was to identify whether a relationship exists between the concentration of HGF and KGF in pulmonary edema and mortality in patients with ALI.

METHODS

Patient Selection and Collection of Samples

The study was approved by the Committee for Human Research at the University of California, San Francisco. A random selection of pulmonary edema fluid and plasma samples was made from patients admitted between 1988 and 1996 to the intensive care units of Moffitt-Long Hospital of the University of California, San Francisco, or to San Francisco General Hospital who had acute respiratory failure and who required mechanical ventilation. Classification of each patient's condition as ALI or hydrostatic pulmonary edema was based on review of medical records, using the North American–European Consensus Conference definitions for ALI (30). Pulmonary edema samples were collected by trained respiratory therapists under the supervision of the authors (M.A.M., G.M.V.), as previously described (6, 31); this method has been well validated experimentally by comparison with transthoracic alveolar aspiration and radionuclide studies (32, 33). All samples were collected within 48 h of development of acute pulmonary edema requiring mechanical ventilation. Briefly, pulmonary edema specimens were obtained by insertion of a soft, size 14 French suction catheter into a wedged position in a distal brochus via the patient's endotracheal tube. Specimens were collected in a Lukens trap and stored at 4° C prior to processing. Pulmonary edema samples were then centrifuged at 14,000 rpm for 20 min, and the supernatant was aspirated and stored at −70° C. Blood samples were collected in ethylenediamine tetraacetic acid (EDTA)-treated tubes within 15 min of pulmonary edema sampling, and centrifuged at 5,000 rpm for 5 min, and the plasma was stored at −70° C.

Measurement of HGF and KGF

Concentrations of hepatocyte and keratinoctye growth factors in undiluted pulmonary edema and plasma specimens were measured by standard enzyme-linked immunosorbent assay (ELISA) technique, using antibodies to recombinant human HGF and KGF. HGF was assayed with a commercial ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN); KGF was measured with an ELISA based on standard techniques, with standards and antibodies provided by Amgen, Inc. (Thousand Oaks, CA). The volume of edema fluid assayed by ELISA was 0.5 ml or greater per sample. Concentrations of HGF and KGF were reported in ng/ml; the lower limit of detection was 0.1 ng/ml.

Partial Purification of HGF and KGF by Heparin–Sepharose Chromatography

HGF and KGF were partly purified from human pulmonary edema samples by heparin–Sepharose chromatography as described previously (10). Briefly, pulmonary edema-fluid specimens from two patients with ALI and two patients with hydrostatic edema were prepared as outlined earlier. A total of 2.5 to 3.5 ml of edema fluid was added to 1 ml of equilibrated heparin–Sepharose beads (Pharmacia Inc., Piscataway, NJ) containing 0.65 ml/ml of heparin–Sepharose. The mixture was gently agitated for 30 min at 4° C and centrifuged at 200 rpm for 2 min. The supernatant was discarded and the beads were washed four times with 5 ml of heparin–Sepharose buffer (Pharmacia Inc., Piscataway, NJ). HGF and KGF were eluted by washing for 15 min with 1 ml of 2.0 M NaCl in heparin–Sepharose buffer; the eluant was collected following passage through Pasteur pipet columns packed with glass wool, and was dialyzed three times against Dulbecco's modified Eagle's medium (DMEM). Positive and negative control solutions of DMEM with 10% fetal bovine serum (DMEM–FBS) and DMEM–FBS:HGF (91 ng/ml):KGF (143 ng/ml) were prepared identically, using recombinant human HGF (R&D Systems) and KGF (Promega, Madison, WI). The final concentrations of HGF and KGF in the purified samples were 8 to 32 ng/ml and 8 to 9 ng/ml, respectively, as measured by ELISA. Final concentrations of both HGF and KGF in the DMEM–FBS control solution were < 0.1 ng/ml, and in the DMEM–FBS:HGF:KGF solution were 72 ng/ml and > 10 ng/ml, respectively.

[^{3}H]Thymidine Incorporation Assay

Bioactivity of HGF and KGF in heparin–Sepharose-purified pulmonary edema-fluid specimens was assessed by incorporation of [^{3}H]thymidine into rat alveolar type II epithelial cells, as previously described (34). Alveolar type II cells were isolated from rats by elastase dissociation and density-gradient centrifugation, according to previously described methods (35). Type II cells were seeded into 48-well tissue-culture plates at a density of 10^5 cells/cm^2, incubated at 37° C for 24 h, and washed with DMEM. Pulmonary edema-fluid and control samples were diluted 1:9 in DMEM with 10% fetal bovine serum (DMEM–FBS). Following this, 0.5 ml of sample solution in DMEM–FBS and 0.1 μCi/ml [^{3}H]thymidine were added to each well. The cells were incubated for 48 h at 37° C and the trichloroacetic acid (TCA)-precipitable counts were measured by liquid scintillation counting. Recombinant human HGF and KGF were used as positive controls. All experiments were done in duplicate.

Experiments to assess neutralization of mitogenic activity by specific neutralizing antibodies to HGF and KGF were done similarly. Anti-HGF and anti-KGF antibodies were obtained from R&D Systems. Alveolar edema-fluid specimens were pooled after partial purification by heparin–Sepharose chromatography. [^{3}H]Thymidine incorporation by type II cells was assayed as previously described, using 5% edema fluid in DMEM–FBS, 5% edema fluid with anti-HGF antibody, and 5% edema fluid with anti-KGF antibody. Positive controls containing recombinant human HGF and KGF at 1, 2, and 5 ng/ml were also assayed. Experiments were done in quadruplicate.

Severity-of-Illness Scores

The Simplified Acute Physiology Score II (SAPS II) (36) and lung injury score (LIS) (37) were calculated for all patients except three patients for whom complete records were unavailable. Data extraction from archived medical records and chest radiograph scoring was done by a single investigator (G.M.V.). Comparisons of SAPS II scores and LIS scores for ALI and hydrostatic edema patients were done with Student's *t* test (two-tailed).

Statistical Analysis

Statistical analysis was done with Statview Student (Abacus Concepts, Inc.) and Microsoft Excel 5.0 (Microsoft Corp., Redwood, WA) software, with statistical significance defined at $p \leq 0.05$. HGF and KGF concentrations are presented as median, 25th to 75th percentiles and 10th to 90th percentiles, since the data were not normally distributed. Data were normalized by log transformation, and were analyzed by analysis of variance (ANOVA). Measurements of [^{3}H]thymidine incorporation were normalized relative to the negative control solution; the data were compared by ANOVA, and Bonferroni's *t* test was used for multiple comparisons.

RESULTS

Patient Characteristics

Twenty-six patients with ALI and 11 patients with hydrostatic pulmonary edema were studied. The patient demographics, etiology of pulmonary edema, and clinical outcomes are detailed in Table 1, with summary statistics in Table 2. The clinical disorders associated with the development of ALI were primary pneumonia (10 of 26 cases), sepsis syndrome (seven of 26 cases), noninfectious pneumonitis (three of 26 cases), neurogenic disorders (two of 26 cases), and other (hemorrhagic shock, postoperative to cardiopulmonary bypass), postoperative to kidney–pancreas transplant, and bowel infarction) (four of 26 cases). The causes of hydrostatic pulmonary edema included acute myocardial infarction and cardiac arrest (four of 11 cases), chronic dilated cardiomyopathy (five of 11 cases), and perioperative volume overload (two of 11 cases). The ratio of protein concentrations in edema fluid and plasma was 0.85 ± 0.27 (mean ± SD) in patients with ALI and 0.56 ± 0.13 in patients with hydrostatic pulmonary edema ($p < 0.05$). SAPS II scores were 53 ± 21 (mean ± SD) and 50 ± 19 in patients with ALI and hydrostatic edema, respectively ($p = 0.65$); the LIS was 3.1 ± 0.5 in ALI and 2.9 ± 0.6 in hydrostatic edema ($p = 0.54$). In-hospital mortality was 73% in the ALI group and 36% in the hydrostatic pulmonary edema group.

TABLE 1

PATIENT CHARACTERISTICS

Study No.	Age (*yr*)	Gender	Diagnosis	Outcome
Hydrostatic				
H05-90	39	M	Cardiac arrest	Expired
H14-93	21	F	SLE, CRT	Survived
H24-88	60	M	Acute MI	Expired
H12-89	81	F	Dilated CM	Survived
H20-89	15	F	Tetralogy of Fallot	Survived
H30-96	64	M	Ischemic CM	Survived
H34-96	45	M	Acute MI	Survived
H50-96	64	M	Ischemic CM	Survived
H22-96	76	M	Ischemic CM	Survived
H06-93	93	M	Ischemic CM	Expired
H24-93	77	F	Acute MI	Expired
Acute lung injury				
H10-94	85	F	Pneumonia	Expired
H06-88	37	F	Methotrexate	Expired
H23-88	30	M	Invasive aspergillosis	Expired
H25-88	24	F	Cerebellar hemorrhage	Survived
H26-88	61	M	Sepsis versus chemotherapy	Expired
H09-89	47	F	Sepsis	Expired
H39-90	88	M	Pneumonia	Expired
H43-90	33	F	Aspiration	Expired
H09-96	82	F	Sepsis	Expired
H22-95	73	M	Pneumonia	Expired
H11-94	40	F	Pneumonia	Survived
H06-93	59	F	Hemorrhagic shock s/p OLT	Expired
H12-94	27	F	Pneumonia	Survived
H08-94	27	M	Sepsis	Expired
H11-93	31	M	Intracranial hemorrhage	Expired
H17-96	38	M	s/p kidney-pancreas transplant	Survived
H20-93	47	M	Bowel infarct	Expired
H04-93	29	M	Pneumonia	Expired
H21-93	35	F	Sepsis	Expired
H28-96	15	M	s/p CPB	Survived
H29-96	30	F	SLE pneumonitis	Survived
H58-96	56	F	Sepsis	Expired
H62-96	48	M	Invasive aspergillosis	Expired
H63-96	60	F	Sepsis	Expired
H64-96	24	M	Pneumonia	Expired
H54-96	77	M	Pneumonia	Survived

Definition of abbreviations: CM = cardiomyopathy; CPB = cardiopulmonary bypass; CRT = cadaveric renal transplant; MI = myocardial infarction; OLT = orthotopic liver transplant; SLE = systemic lupus erythematosus; s/p = status post-.

TABLE 2

SUMMARY OF PATIENT CHARACTERISTICS

	n	Age (Range) (*yr*)	Gender	SAPS II	LIS
Hydrostatic HGF	11	58 (21–93)	64% M	50 ± 19	2.9 ± 0.5
Hydrostatic KGF	6	70 (45–93)	83% M	61 ± 17	3.0 ± 0.6
ALI HGF	26	46 (15–88)	50% M	53 ± 21	3.1 ± 0.5
ALI KGF	25	47 (15–88)	52% M	53 ± 21	3.1 ± 0.5

Definition of abbreviations: ALI = acute lung injury; HGF = hepatocyte growth factor; KGF = keratinocyte growth factor; LIS = lung injury score; SAPS II = Simplified Acute Physiology Score II.

Data expressed as mean ± SD.

HGF Concentrations in Pulmonary Edema and Plasma

HGF concentrations in edema fluids from patients with ALI ranged from 1.8 to 148 ng/ml, with a median of 21.4 ng/ml (8.3 to 41.3 ng/ml) (25th to 75th percentiles); HGF in pulmonary edema fluid from the patients with hydrostatic edema ranged between 2.4 and 34.5 ng/ml, with a median concentration of 6.6 ng/ml (4.8 to 11.4 ng/ml) (25th to 75th percentiles) (Table 3). The concentration of HGF was significantly higher in the pulmonary edema fluids from patients with ALI than in those from the hydrostatic edema group ($p < 0.01$) (Figure 1A). HGF was detected in plasma from both groups of patients, but there was no significant difference in HGF concentration between the two groups (Figure 1B). In both groups of patients, the level of HGF in pulmonary edema fluid was significantly greater than in plasma ($p < 0.01$ for ALI; $p < 0.01$ for hydrostatic edema). The correlation coefficients between edema fluid and plasma HGF levels were 0.56 ($p < 0.01$) for the ALI group and 0.79 ($p < 0.01$) for the hydrostatic edema group (data not shown).

TABLE 3

HEPATOCYTE GROWTH FACTOR AND KERATINOCYTE GROWTH FACTOR CONCENTRATIONS IN PULMONARY EDEMA FLUID AND PLASMA

Clinical Condition	HGF (*ng/ml*)	KGF (*ng/ml*)
ALI edema	21.4 (8.3–41.3)	0.6 (0.3–2.1)
ALI plasma	3.1 (1.8–5.0)	0.3 (0.2–0.7)
Hydrostatic edema	6.6 (4.8–11.4)	0.2 (0.0–2.6)
Hydrostatic plasma	1.7 (1.0–4.4)	0.1 (0.1–0.2)

Definition of abbreviations: ALI = acute lung injury; HGF = hepatocyte growth factor; KGF = keratinocyte growth factor.

Data expressed as median with 25th to 75th percentiles.

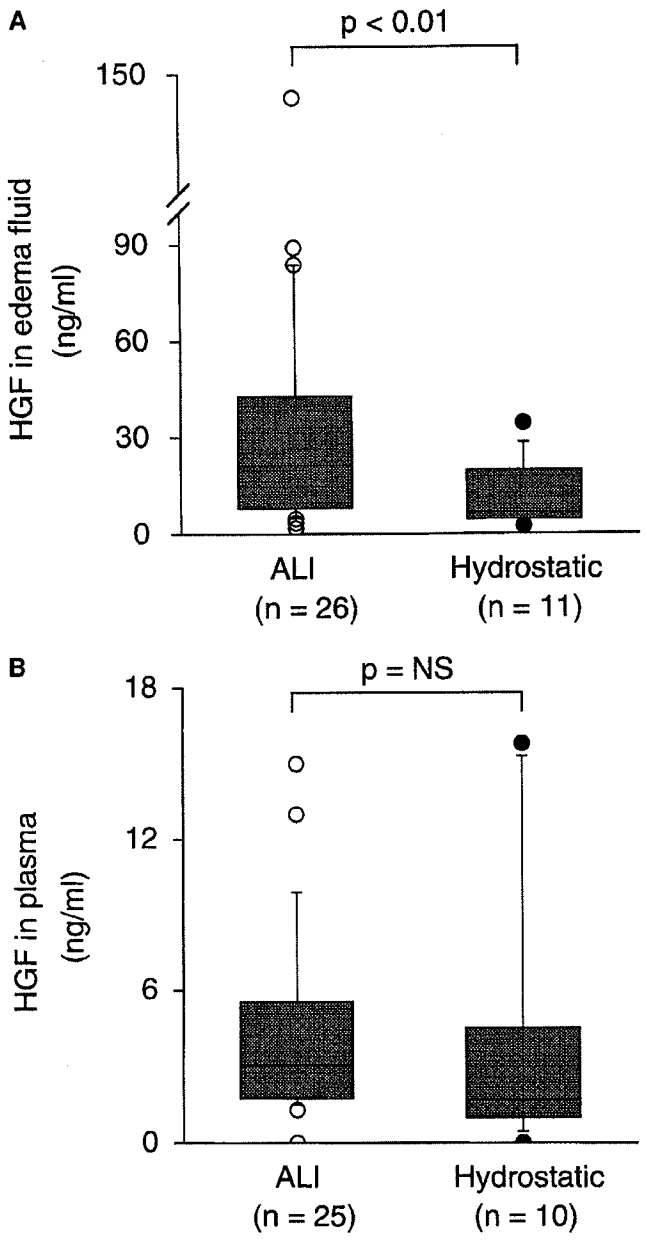

Figure 1. HGF concentrations in undiluted pulmonary edema fluid (*A*) and plasma (*B*) samples from patients with ALI or hydrostatic pulmonary edema. HGF measurements were made with an ELISA. *Box plots* represent the median and 25th to 75th percentiles; *error bars* span 10th to 90th percentiles. Analysis was done by ANOVA of log-transformed data.

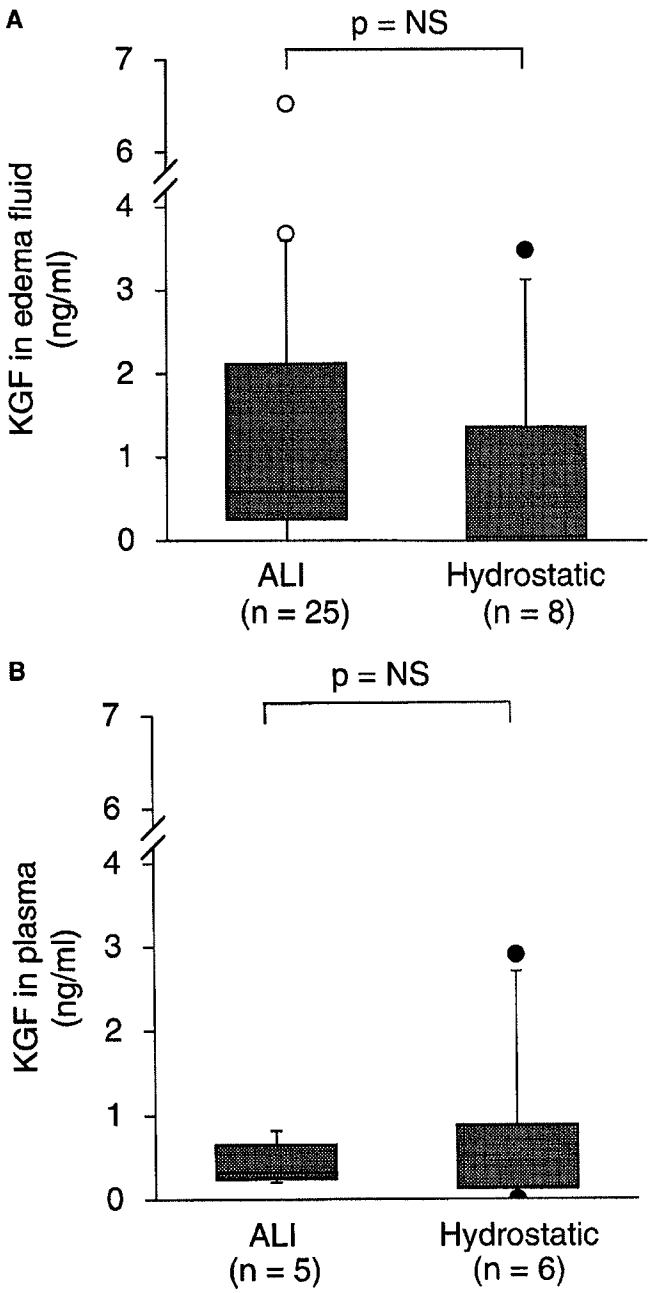

Figure 2. KGF concentrations in undiluted pulmonary edema (*A*) and plasma (*B*) samples from patients with ALI or hydrostatic pulmonary edema. KGF measurements were made with an ELISA. *Box plots* represent the median and 25th to 75th percentiles; *error bars* span 10th to 90th percentiles. Analysis was done by ANOVA after normalization of data by logarithmic transformation.

KGF Concentrations in Pulmonary Edema and Plasma

KGF was detectable in pulmonary edema fluid and plasma from patients with ALI and hydrostatic pulmonary edema (Figure 2). The median (25th to 75th percentiles) concentrations of KGF in pulmonary edema fluid were 0.59 ng/ml (0.26 to 2.13 ng/ml) in patients with ALI/ARDS, compared with 0.21 ng/ml (0.00 to 2.60 ng/ml) in patients with hydrostatic pulmonary edema (*see* Table 3). There were no statistically significant differences between KGF concentrations in pulmonary edema fluid or plasma of patients with ALI and those with hydrostatic edema. There was no significant correlation between the concentrations of KGF detected in edema fluid and plasma in either group (data not shown).

Bioactivity of HGF and KGF in Pulmonary Edema

In order to determine whether the growth factors detected by ELISA were biologically active, HGF and KGF in pulmonary edema samples from two patients with ALI and two patients with hydrostatic edema were partly purified by heparin–Sepharose chromatography. The activity of HGF and KGF

from these specimens was assessed by their ability to stimulate DNA synthesis in primary cultures of rat alveolar type II cells. DNA synthesis was measured by incorporation of [^{3}H]thymidine into the type II cells during 48 h of incubation with partly purified pulmonary edema fluids. The HGF concentration in the purified samples ranged from 5 to 32 ng/ml; KGF concentrations were between 8.2 and 9.0 ng/ml. As shown in Figure 3, [^{3}H]thymidine incorporation in cells treated with the edema fluids increased by 6.3- to 9.2-fold as compared with the DMEM–FBS control ($p = 0.0039$ for Bonferroni's t test), which was similar in magnitude to the effect of high concentrations of recombinant human HGF and KGF (72 ng/ml and > 10 ng/ml, respectively) ($p = 0.102$ for Bonferroni's t test). Pooled, partly purified edema fluid was diluted in DMEM–FBS to 1%, 2%, 5%, and 10% solutions; a dose–response curve for stimulation of DNA synthesis showed maximum [^{3}H]thymidine incorporation with 5% edema fluid (data not shown). As shown in Figure 4, a 5% edema-fluid solution stimulated [^{3}H]thymidine incorporation by 5.8-fold as compared with the control ($p = 0.002$). Addition of anti-HGF antibody reduced DNA synthesis by 66% ($p = 0.003$); addition of anti-KGF antibody diminished DNA synthesis by 53% ($p = 0.009$). Thus, most of the DNA synthesis stimulated by heparin-binding growth factors in the pulmonary edema fluids was due to HGF and KGF.

Relationship Between Mortality and Pulmonary Edema HGF and KGF Concentrations

The in-hospital mortality rate among the patients with ALI was 73%. In the ALI group, the median (25th to 75th percentiles) HGF concentration in edema fluid of the seven survivors was 6.5 (4.1 to 13.4) ng/ml, whereas the HGF was 27.3 (11.3 to 44.8) ng/ml in the 19 patients who expired (Figure 5). The patients with ALI who survived had significantly lower levels of HGF in their pulmonary edema fluid than did those who expired ($p = 0.03$). Similar analysis of the data for patients with hydrostatic pulmonary edema showed no significant differences in the edema-fluid HGF concentration between survivors and nonsurvivors (data not shown).

Overall hospital mortality was 76% for the group of patients with ALI in whom pulmonary edema-fluid KGF was measured ($n = 25$). The median (25th to 75th percentiles) KGF in pulmonary edema fluid sampled from patients with ALI was 0.4 (0.3 to 0.6) ng/ml in survivors and 1.1 (0.1 to 2.8) ng/ml in the nonsurvivors (data not shown). There was no significant difference in edema-fluid KGF level in survivors and nonsurvivors ($p = 0.13$). Equivalent analysis of the data for the patients with hydrostatic pulmonary edema revealed no significant association between edema-fluid KGF concentration and mortality.

DISCUSSION

HGF was present in both the pulmonary edema fluid and plasma from patients with severe ALI and those with hydro-

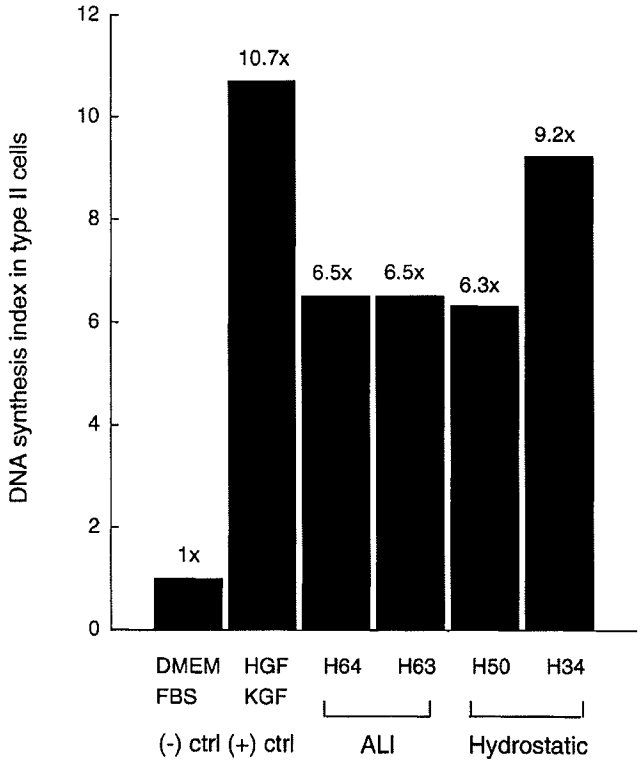

Figure 3. Bioactivity of HGF and KGF in edema fluids. HGF and KGF were partly purified from edema fluids obtained from two patients with ALI and two patients with hydrostatic edema by heparin–Sepharose chromatography and elution with 2.0 M NaCl. The negative and positive controls were DMEM–FBS and recombinant human HGF (72 ng/ml) and recombinant human KGF (10 ng/ml). HGF concentrations ranged from 5 to 32 ng/ml and KGF concentrations from 8.2 to 9.0 ng/ml in the purified samples. *Bars* represent the relative uptake of [^{3}H]thymidine by primary cultures of rat type II pneumocytes.

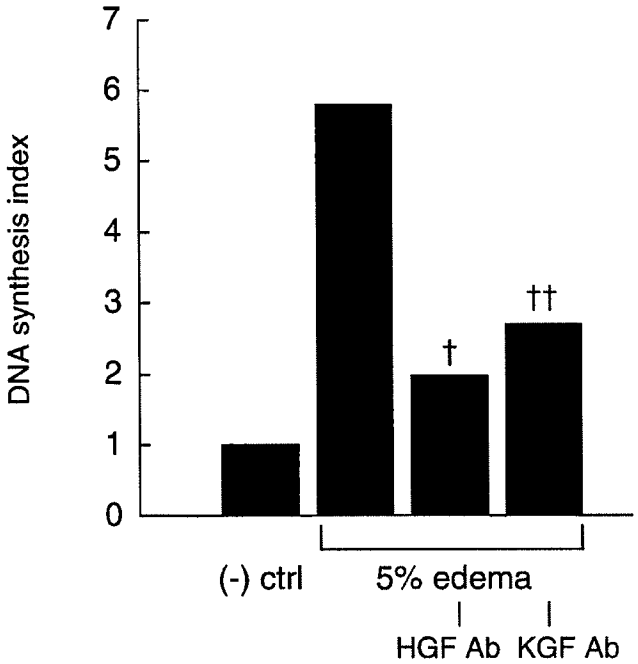

Figure 4. Antibody neutralization of DNA synthesis stimulated by pulmonary edema fluid. Anti-HGF and anti-KGF, neutralizing antibodies to recombinant human HGF and KGF, were added to a 5% solution of pooled pulmonary edema fluid samples that were partly purified by heparin–Sepharose chromatography. The DNA synthesis index is a measure of [^{3}H]thymidine incorporation by primary cultures of rat type II alveolar epithelial cells treated with edema fluid and relative to [^{3}H]thymidine incorporation by cells of the DMEM-FBS-treated control. †, †† Statistically significant differences compared with the 5% pulmonary edema fluid.

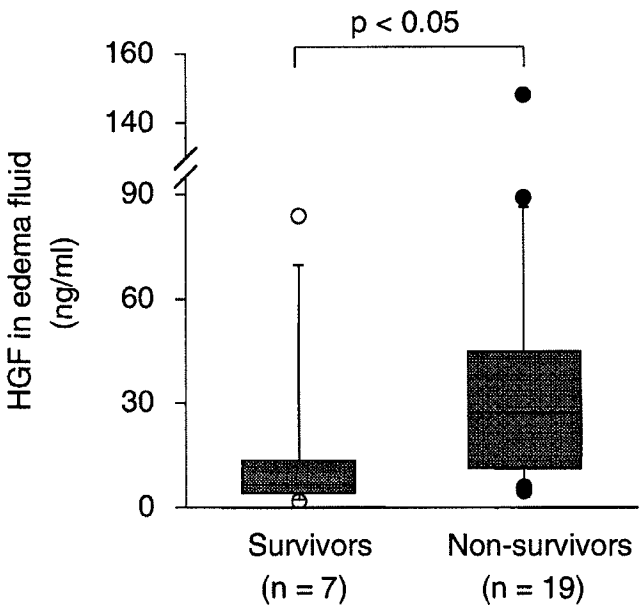

Figure 5. Relationship between pulmonary edema-fluid levels of HGF and mortality in patients with ALI. Analysis was done by ANOVA of data normalized by log transformation.

static pulmonary edema. HGF was detected in a significantly higher concentration in the pulmonary edema fluid of patients with ALI than of those with hydrostatic edema. The primary source of HGF in these patients seems to have been the lung, since the HGF level was 7-fold higher in the pulmonary edema fluid than in the plasma. Additionally, the heparin-binding growth factors recovered from these pulmonary edema-fluid specimens, primarily HGF and KGF, were biologically active, as indicated by their ability to stimulate DNA synthesis in isolated rat alveolar type II cells.

The concentrations of HGF in plasma from patients with ALI and acute hydrostatic pulmonary edema were much higher than the concentrations measured in prior studies of patients with other inflammatory lung diseases. Yanagita and colleagues found plasma HGF concentrations ranging from 1.1 to 2.8 ng/ml in seven hospitalized patients with bacterial pneumonia (23), and Maeda and coworkers reported similar findings in patients with active interstitial pneumonitis (1.2 ± 0.2 ng/ml, n = 15) and bacterial pneumonia (1.0 ± 0.3 ng/ml, n = 11) (22). Thus, the median levels of HGF in plasma from these patients with ALI were approximately 3-fold greater than those in plasma from patients with other active pulmonary diseases. The higher levels of HGF most likely reflect a greater degree of injury and inflammation in the ALI patients. Indeed, the high plasma concentrations of HGF are similar to those measured in patients with other types of major organ failure, such as fulminant hepatic failure and severe acute pancreatitis, in whom the mean serum HGF concentrations were 8.1 ± 1.8 ng/ml and 3.2 ± 1.7 ng/ml, respectively (19, 20). The concentrations of HGF measured in plasma and pulmonary edema-fluid specimens are within the range of maximal HGF activity of approximately 1 to 10 ng/ml, on the basis of *in vitro* studies of DNA synthesis by rat alveolar type II cells (8).

The conclusion that the high levels of HGF found in these patients with severe lung injury is a marker for severe inflammation is supported by clinical studies of interstitial pneumonitis and bacterial pneumonia, fulminant hepatic failure, and acute pancreatitis, in all of which a strong positive correlation was shown between the serum HGF concentration and physiologic markers of organ damage (19, 20, 22). This explanation is also supported by *in vitro* and *in vivo* studies of the regulation and cellular effects of HGF. Expression of HGF is upregulated by several proinflammatory cytokines, including interleukin-1α (IL-1α), IL-1β, and tumor necrosis factor-α (TNF-α) (38), which are important in the neutrophilic infiltration occurring in ALI and are present in the alveolar space early in lung injury (39–42). HGF is a potent mitogen for alveolar type II cells *in vitro* and *in vivo*, and is upregulated within 6 h of acid-instillation into rat lungs (23). Furthermore, intravenous administration of HGF to mice after induction of ALI by acid instillation stimulated DNA synthesis in alveolar type II cells (43). These experimental results suggest that expression of HGF, probably by pulmonary interstitial fibroblasts (10) and endothelial cells (44), is upregulated by proinflammatory cytokines early in ALI, with the potential benefit of stimulating proliferation of type II cells and restoring the alveolar epithelial barrier. This mechanistic explanation is further strengthened by our findings of physiologic concentrations of bioactive HGF in pulmonary edema fluid from patients with ALI. Other possible contributors to the HGF measured in the plasma of these critically ill patients are nonpulmonary sources, including the liver and kidney in patients with multiple organ damage, or increased pulmonary expression and release of HGF in response to injury of distant organs, as reported in studies of partial hepatectomy and nephrectomy in rats (45). However, these are probably relatively minor factors in view of our findings that the level of HGF in pulmonary edema fluid was 7-fold higher than in the plasma of patients with ALI, and that pulmonary edema and plasma concentrations of HGF were positively correlated, both of which findings suggest that the lung is the major source of HGF in these patients.

In this study, the concentration of HGF in pulmonary edema fluid from patients with ALI was significantly higher in patients who expired prior to hospital discharge than in those who survived. This finding concurs with those in studies of fulminant hepatitis, acute pancreatitis, and inflammatory lung diseases not including ALI, in which progressively increasing levels of serum HGF were found in patients who expired (19, 20, 22). The relationship between mortality and higher HGF levels in pulmonary edema fluid probably represents an intense reparative response to severe lung injury. Although these data should be confirmed in a larger prospective study using predetermined thresholds of HGF concentrations, the correlation between pulmonary edema fluid HGF concentrations and mortality may be a clinically useful marker of prognosis in ALI for several reasons. Most importantly, the HGF concentration measured very early in the course of ALI correlates with mortality; additionally, pulmonary edema fluid is easily sampled from mechanically ventilated patients, and HGF can be measured with ELISA techniques available to most clinical laboratories. This measurement may also be a useful adjunct for identifying those patients at highest risk for nonsurvival, for the purposes of clinical studies of ALI, and perhaps when assessing the relative benefit of providing experimental therapies for ALI.

In contrast to HGF, KGF was present only in small quantities in the edema fluid and plasma of patients with ALI or hydrostatic pulmonary edema. Furthermore, there was no significant difference between the edema fluid or plasma levels of KGF in ALI and hydrostatic edema (Figure 2). Nevertheless, the present study is the first in which KGF was detected in biologic fluids from patients. Are these levels of KGF biologi-

cally significant? Large quantities of exogenously administered KGF (1 to 5 mg/kg) are necessary to induce type II-cell hyperplasia and to attentuate lung injury *in vivo* in normal rats (24–27). However, on the basis of *in vitro* data, KGF concentrations of only 10 ng/ml are required to maximally stimulate alveolar type II-cell proliferation and surfactant protein gene expression (10, 46). Thus, the concentrations of KGF found in pulmonary edema fluid may be biologically active. Other factors, such as KGF-receptor activation, will also determine the biologic effects of these concentrations of KGF.

Although pretreatment of rats with intratracheal KGF diminishes the severity of ALI caused by a variety of experimental insults, the role of endogenous KGF in recovery from ALI is not understood. In addition to its mitogenic activity, KGF stimulates expression of surfactant proteins and production of lamellar bodies in type II pneumocytes *in vitro* and *in vivo*, indicating that KGF may promote differentiation of these cells (46, 47). These data lead to the hypothesis that KGF may be an important mediator of type II-cell hyperplasia and maturation early in the response to ALI, resulting in restoration of the denuded alveolar epithelium and replenishment of surfactant. This would represent an effect similar to that in dermal wound healing, in which KGF is induced in fibroblasts at the wound site, and stimulates both proliferation and migration of keratinocytes at the wound edge (48, 49). Expression of KGF by fibroblasts is upregulated by IL-1α, IL-1β, TNF-α, IL-6, and transforming growth factor-α (TGF-α) (50, 51), cytokines that have been identified in bronchoalveolar lavage fluid (BALF) and pulmonary edema specimens from patients with ARDS (39–42, 52). Furthermore, KGF is known to be secreted by human pulmonary fibroblasts (10). Our findings of detectable levels of bioactive KGF in pulmonary edema fluid from patients early in the course of ALI is consistent with this proposed sequence of events, lending further support to a role for KGF in recovery of the alveolar epithelial barrier following lung injury.

Why was mortality 73% in the 26 ALI patients with pulmonary edema fluid sampled in the present study, as compared with 58% in our recently published study of 123 consecutive patients with ALI treated in the same intensive care unit (53)? The most likely explanation is that the quantity of edema fluid is greater in patients with more severe lung injury, and that the method of obtaining direct aspirates of alveolar edema fluid therefore selects a group of patients with more severe ALI. In support of this hypothesis, we have recently reported a mortality of 64% in ALI patients from whom edema fluid was sampled for another study (54). Thus, the results of these studies should not be generalized to all patients with ALI.

An interesting and unexpected finding in the present study was the presence of both HGF and KGF in pulmonary edema fluid and plasma from patients with severe hydrostatic pulmonary edema. The median plasma (25th to 75th percentiles) HGF concentration in this group was 1.7 (1.0 to 4.4 ng/ml), which was much higher than the average of approximately 0.3 ng/ml reported in the literature for normal control subjects (20, 22, 23). Three of these patients had very high levels of HGF measured in pulmonary edema-fluid samples, ranging from 24 to 35 ng/ml, which were more consistent with the values found in the patients with ALI. One explanation for the presence of high levels of HGF and detectable KGF in plasma and pulmonary edema fluid from these patients with hydrostatic edema is the increased production of cytokines in congestive heart failure (CHF). Other possibilities include stimulation of cytokine production by plasma leaking into the interstitial space, and stretching of alveolar structures by positive-pressure ventilation (55). Several clinical studies have found increased concentrations of TNF-α and IL-6 in plasma, which correlated with the severity of CHF (56, 57), although Ferrari and associates also found that the concentration of soluble TNF receptor was increased, and that the overall cytotoxic activity of TNF was no different than in controls (58). Hennein and colleagues (59) have reported that TNF-α, IL-6, and IL-8 can be increased after uncomplicated coronary artery bypass grafting in correlation with the duration of aortic cross-clamping, and that increasing levels of IL-6 and IL-8 were associated with postoperative left ventricular dysfunction. These findings show that the cytokine milieu is enhanced in the serum of patients with left ventricular dysfunction or injury, an effect that may lead to release of HGF and KGF through the regulatory pathways discussed earlier. These mechanisms may even be important mediators of the alveolar epithelial type II-cell hyperplasia observed morphologically in chronic, severe, CHF (60); this response could increase the capacity for alveolar-fluid transport in the presence of chronic heart failure (61).

The need to include control patients with hydrostatic pulmonary edema in measuring levels of HGF and KGF in ALI is evident from the present study. First, the presence of HGF and KGF in alveolar edema fluid is not specific for ALI, but is common to pulmonary edema of all sources. Second, significant quantities of HGF are released in some patients with hydrostatic pulmonary edema, indicating that some patients with hydrostatic pulmonary edema may have a modest proinflammatory environment. These findings underscore the importance of studying hydrostatic pulmonary edema in addition to ALI when investigating potential biomarkers of injury, in order to control for the presence of pulmonary edema and the effects of positive-pressure ventilation. Importantly, the SAPS II and LIS were similar in patients with pulmonary edema resulting from increased permeability or hydrostatic mechanisms (Table 2).

In summary, HGF and KGF are present and biologically active in the pulmonary edema fluid of patients with ALI. Furthermore, higher concentrations of HGF are associated with mortality in patients with ALI. Combined with experimental data, the findings of this study strengthen the hypothesis that both HGF and KGF are induced by proinflammatory cytokines early in lung injury, and may work in concert to restore damaged alveolar epithelium by promoting the proliferation and differentiation of alveolar type II epithelial cells. Of clinical importance is that the degree of increase of HGF in edema fluid may reflect the severity of injury, and may therefore be a useful prognostic marker for poor outcome in ALI.

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Hepatocyte growth factor is elevated in chronic lung injury and inhibits surfactant metabolism

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Vivekananda, Jeevalatha, Vibhudutta Awasthi, Shanjana Awasthi, Dolphin B. Smith, and Richard J. King. Hepatocyte growth factor is elevated in chronic lung injury and inhibits surfactant metabolism. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 278: L382–L392, 2000.— Adult respiratory distress syndrome may incorporate in its pathogenesis the hyperplastic proliferation of alveolar epithelial type II cells and derangement in synthesis of pulmonary surfactant. Previous studies have demonstrated that hepatocyte growth factor (HGF) in the presence of serum is a potential mitogen for adult type II cells (R. J. Panos, J. S. Rubin, S. A. Aaronson, and R. J. Mason. *J. Clin. Invest.* 92: 969–977, 1993) and that it is produced by fetal mesenchymal lung cells (J. S. Rubin, A. M.-L. Chan, D. P. Botarro, W. H. Burgess, W. G. Taylor, A. C. Cech, D. W. Hirschfield, J. Wong, T. Miki, P. W. Finch, and S. A. Aaronson. *Proc. Natl. Acad. Sci. USA* 88: 415–419, 1991). In these studies, we expand on this possible involvement of HGF in chronic lung injury by showing the following. First, normal adult lung fibroblasts transcribe only small amounts of HGF mRNA, but the steady-state levels of this message rise substantially in lung fibroblasts obtained from animals exposed to oxidative stress. Second, inflammatory cytokines produced early in the injury stimulate the transcription of HGF in isolated fibroblasts, providing a plausible mechanism for the increased amounts of HGF seen in vivo. Third, HGF is capable of significantly inhibiting the synthesis and secretion of the phosphatidylcholines of pulmonary surfactant. Fourth, HGF inhibits the rate-limiting enzyme in de novo phosphatidylcholine synthesis, CTP:choline-phosphate cytidylyltransferase (EC 2.7.7.15). Our data indicate that fibroblast-derived HGF could be partially responsible for the changes in surfactant dysfunction seen in adult respiratory distress syndrome, including the decreases seen in surfactant phosphatidylcholines.

phosphatidylcholine metabolism; protein kinase C; protein kinase A; cytidine 5′-triphosphate:choline-phosphate cytidylyltransferase; adult respiratory distress syndrome

THE ONGOING SYNTHESIS AND SECRETION of pulmonary surfactant, a complex lipoprotein produced by alveolar epithelial type II cells, is necessary for normal lung function. Previous studies indicate that the amounts and composition of pulmonary surfactant are perturbed in adult respiratory distress syndrome (ARDS) (10) and bronchopulmonary dysplasia (18, 25), chronic lung injuries in adults and premature newborns, respectively. The etiologies of both syndromes frequently involve reparative processes characterized by increased populations of interstitial fibroblasts and alveolar epithelial cells, and the latter are frequently altered in appearance. The identity of substances responsible for these actions is uncertain, although growth factors are widely proposed as likely candidates.

Paracrine-autocrine regulation involving feedback loops between mesenchymal and epithelial cells is becoming increasingly recognized for its importance in fetal development and injury repair (32). Specifically among lung cells, Brody (5) has demonstrated that mesenchymal-epithelial contacts through pseudopods increase with fetal development, and Adamson et al. (2) have found similar contacts in chronic lung injury in adults. Fibroblasts synthesize several growth factors, including hepatocyte growth factor (HGF), that might affect these changes in surfactant synthesis and cell proliferation (32). HGF is a multifunctional peptide originally recognized for its effects on cell proliferation and its ability to affect cell motility and aggregation. More recent studies (43a) indicate a far wider range of actions, and it may also participate in malignancy, wound repair, and neovascularization concomitant with inflammatory disease. HGF is produced by mesenchymal cells, but they do not contain its receptor, c-met. In contrast, epithelial cells contain the receptor but do not produce the ligand. Thus signaling is exclusively by mesenchymal-epithelial communication (32).

Several reports (27, 38) show that HGF in combination with serum is a mitogen for alveolar type II cells in primary culture, a property that it shares with keratinocyte growth factor (27), acidic fibroblast growth factor (38), and transforming growth factor-α (34). HGF is produced by cloned cell lines of fetal lung fibroblasts (27, 33), and Yanagita et al. (48) have demonstrated that steady-state levels of HGF mRNA are transiently increased in response to an intratracheal injection of HCl. Here we present evidence that HGF may be involved not only in the stimulated proliferation of lung cells in ARDS but also in the reduction of alveolar pools of surfactant. First, we show that oxidative stress leading to chronic lung injury results in a sustained transcription of HGF (compared with the temporary increase found in the protocol of Yanagita et al.). The likely source of this increased HGF is lung fibroblasts.

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Second, cytokines that are produced in response to lung injury stimulate the transcription of HGF in lung fibroblasts. Third, HGF inhibits both the synthesis and secretion of phosphatidylcholine (PC) when presented to isolated type II cells. Furthermore, we present limited evidence on the mechanisms of these actions by HGF. We find that HGF inhibits the activity of the rate-limiting enzyme in PC synthesis, CTP:cholinephosphate cytidylyltransferase (CT; EC 2.7.7.15), possibly acting through a proline-dependent kinase. We are unable, however, to demonstrate the involvement of protein kinase (PK) C in inhibiting secretion.

METHODS

Animal procedures. Adult rats were exposed to 100% oxygen at 1 atm, as described (23). In brief, adult rats (175–200 g) were exposed to 100% oxygen for up to 10 days in a stainless steel-Plexiglas chamber with access to water and food ad libitum. Oxygen was delivered at a rate of 15 ml/min and recirculated after being scrubbed for carbon dioxide. Each day the chamber was opened and cleaned, and the animals were aired for 1.5 h in the morning and late afternoon. Rats exposed continuously to 100% oxygen die within 72 h; exposure to room air extends longevity to a time course more consistent with chronic lung injuries in primates. At the end of each exposure period, the animals were removed from the chamber, transported to the laboratory, and killed for cell isolation or biochemical studies.

Animal protocols were approved by the Institutional Animal Care and Use Committee, and all animals used were cared for in accordance with institutional guidelines.

Fibroblast cell culture. Lung tissue was dissociated with 2 mg/ml trypsin (type II; Sigma) and 0.16 mg/ml DNase 1 (Sigma) in MEM for 20 min at 37°C. The cell suspensions were treated with 10% FCS to stop the enzymatic reaction, and the cells were plated in MEM containing 10% FCS, 2.5 μg/ml of Fungizone, and 50 μg/ml of gentamicin. After 60 min at 37°C, the culture medium containing floating cells was removed and replaced with fresh MEM. The adherent fibroblasts used in these studies were passaged three times and displayed a typical fibroblast morphology when examined with inverted phase microscopy. Macrophages or monocytes were not evident.

Culture of NCI-H441 cells. The cells were obtained from American Type Culture Collection as batch F-12518 of the 50th passage. The cells were maintained in McCoy's 5A medium with 10% FCS and 50 μg/ml gentamicin.

Isolation of alveolar type II cells. As described by Dobbs et al. (12), type II cells from normal adult rats were isolated as follows: lungs were perfused with DMEM, lavaged with a calcium-balanced salt solution, and filled with 12 U/ml of pancreatic elastase (Worthington). Tissue was dissociated by incubation at 37°C for 20–30 min. Cells were filtered through gauze pads, washed, and incubated on microbiological plastic coated with rat IgG. Nonadherent cells were recovered and washed by centrifugation, and 1–2 million cells/well were placed in 24-well plates on uncoated inserts (Millipore) for study of surfactant metabolism. Purity, as assessed by staining with Papanicolaou reagents, was generally ~85% type II cells, with the populations ranging from 80 to 90%.

Study of surfactant metabolism. All experiments were conducted in a defined medium consisting of DMEM containing 10^{-4} M hydrocortisone, 5×10^{-4} M cAMP, 1 μM/ml insulin-transferrin-selenium (Collaborative Research), and 6 μM arginine-glycine-aspartic acid-serine (Sigma). No FCS was given to the cells at any time during the experiments.

Cells were incubated in phosphate-free defined medium for 1 h. Medium was then removed, and the cells were given 50 μCi/well of [^{32}P]orthophosphate in the same defined medium, together with either rat HGF (5 ng/ml) or solvent. In separate experiments testing the effects of inhibiting tyrosine kinases, cells received HGF plus sodium orthovanadate (SOV; 20 μM) or SOV alone. Cells were incubated for up to 48 h at 37°C in a CO_2 incubator, and at varying times, medium was removed from the wells and cells were recovered from the inserts by gentle trituration. After a wash in DMEM, the cells were suspended in 2 ml of H_2O and lysed by freeze-thaw. PCs were isolated with the following protocol. Lipids in cells and medium were extracted and dissolved in a small volume of 1:1 (vol/vol) chloroform-methanol, and the phospholipids were separated by silica gel thin-layer chromatography as previously described (23). Phospholipids were detected by a 2-(*p*-toluidino)naphthalene-6-sulfonic acid (TNS) spray, and the PC band was recovered by scraping and transesterified with 1% H_2SO_4 in methanol for 2 h at 70°C together with an internal standard of a known amount of diheptadecanoyl PC. Methyl esters were extracted with hexane, and PC content was quantified by gas-liquid chromatography. The lower phase of the extract containing the radioactive glycerolphosphocholine was used to quantify the incorporation of orthophosphate by scintillation counting.

Extraction and analysis of RNA. Steady-state levels of HGF mRNA in lung fibroblasts were determined by Northern blots or RNase protection assay (RPA). Confluent monolayers were lysed with guanidinium isothiocycanate, and total RNA was isolated as described by Chomczynski and Sacchi (9). For Northern blots, ~50 μg of total RNA were applied for electrophoresis in 1% agarose-formaldehyde gels and transferred to 0.45 μM Nytran in 10× sodium chloride-sodium citrate (0.15 M NaCl and 15 mM $Na_3C_6H_5O_7$, pH 7.0). A 1.4-kb cDNA fragment of rat HGF (41) coding for portions of the α- and β-chains was labeled with [^{32}P]dCTP with a random-labeling kit (Pharmacia). Hybridization was performed overnight at 42°C using $\sim 1 \times 10^6$ counts·min^{-1} (cpm)·ml^{-1} of labeled cDNA. The blot was washed for 15 min once with 0.1% sodium dodecyl sulfate (SDS) in 0.1× sodium chloride-sodium phosphate-EDTA (SSPE; 0.15 M NaCl, 10 mM Na_3PO_4, and 1 mM EDTA, pH 7.4), followed by two washes with 0.1% SDS in 6× SSPE. The radioautographs were evaluated by comparison with the total amount of loaded RNA and quantified by staining with ethidium bromide and gel scanning.

RPA was done with an RPA II kit supplied by Ambion following the manufacturer's recommended procedures. A 200-bp antisense cRNA probe to nucleotides 1626–1827 of rat HGF was synthesized by RT-PCR and a Maxiscript kit (Ambion) with RNA isolated from rat liver. The probe was sequenced, and the sequence was confirmed to be identical to authentic rat HGF (41).

Assay of PKA activity. Analyses were conducted with commercially available kits following instructions supplied by the manufacturer (GIBCO BRL, Life Technologies). Briefly, 2–6 million cells were incubated in culture with 5 ng/ml HGF or with the buffer control for 1–2 h with the culture conditions described in *Study of surfactant metabolism.* The cells were recovered, washed by centrifugation, and extracted with 20 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, and protease inhibitors. PKA activity was assayed directly from the lysates using a PKA-specific activator as described in the kit.

Assay of PKC activity. H441 cells were washed twice with ice-cold PBS and scraped in 200 μl of homogenization buffer [20 mM Tris·HCl (pH 7.5), 0.25 M sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml of leupeptin, 25 μg/ml of pepstatin, 1 mM

phenylmethylsulfonyl fluoride, and 0.2 mM SOV]. To separate cytosolic and membrane fractions, the cells were homogenized and centrifuged at 40,000 rpm for 30 min at 4°C (Beckman). The membrane pellet was dissolved in 100 μl of homogenization buffer containing 0.05% Triton X-100, kept on ice for 30 min, and centrifuged as above. Cytosolic and solubilized-membrane fractions (10 μl each) were incubated with 2 μg of myelin basic protein peptide, acids 4–14 (MBP_{4-14}), 0.8 μg/ml of diolein, 8 μg/ml of phosphatidylserine, 20 μM ATP, and 1 μCi of [^{32}P]ATP in 50 μl of kinase buffer [20 mM Tris·HCl (pH 7.5), 10 mM magnesium acetate, 1 mM $CaCl_2$, and 50 μg/ml of leupeptin]. The peptide substrate MBP_{4-14} has been shown to be resistant to phosphatase digestion (13) and is a specific substrate for PKC (50). The reaction was allowed to proceed for 15 min at 30°C. Aliquots (10 μl) of the reaction mixtures were applied to phosphocellulose paper disks, and the disks were washed three times with 1% acetic acid and twice with water before counts were measured in a scintillation counter. Nonspecific activity in the cytosolic and membrane fractions was estimated by subtracting activity after phosphatidylserine and diolein were omitted and 1 mM EDTA was substituted for $CaCl_2$. Activities were calculated as the amount of phosphorylation of MBP_{4-14} per microgram of cell protein per minute.

Quantification of CT activity. Activity was measured as described by Vance et al. (43) as the amount of [^{14}C]phosphocholine converted to [^{14}C]CDPcholine. Approximately 2 million H441 cells were placed overnight in McCoy's 5A medium without FCS. The following morning, the cells were washed and given new medium containing 10 ng/ml of human HGF (R&D Systems) or equivalent volumes of control medium. Cells were recovered at various times after HGF was provided, washed twice, and assayed for CT activity. Results of different experiments were compared by expressing data as percent of control activity at each sampling time. Similar procedures were followed with freshly isolated type II cells placed overnight in DMEM to allow stabilization and treated the next morning with 10 ng/ml of HGF.

Quantitation of diacylglycerol content. H441 cells were plated in 12-well plates in McCoy's 5A medium with 10% FCS. The morning of the experiment, the cells were washed twice with medium without FCS and equilibrated for 1 h in fresh McCoy's without FCS containing ~100,000 cpm of [9,10-^{3}H] palmitic acid. HGF was added after 1 h (*time 0* for the timing of the experiment), and cells were harvested at varying times after isotope was added. Cells were washed twice and scraped into 0.8 ml of H_2O. Lipids were extracted and spotted together with 1,2-diacylglycerol (DAG) on silica gel thin-layer plates. The plates were developed with a solvent system of ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2), followed by ethyl ether-hexane (6:94) (14). 1,2-DAG was clearly separated from 1,3-DAG and cholesterol in this solvent system. The lipids were visualized by spraying with TNS, and the 1,2-DAG spot was scraped and counted. Time-matched control experiments were run at all times, and changes in 1,2-DAG induced by HGF were quantified by differences from these time-matched control experiments.

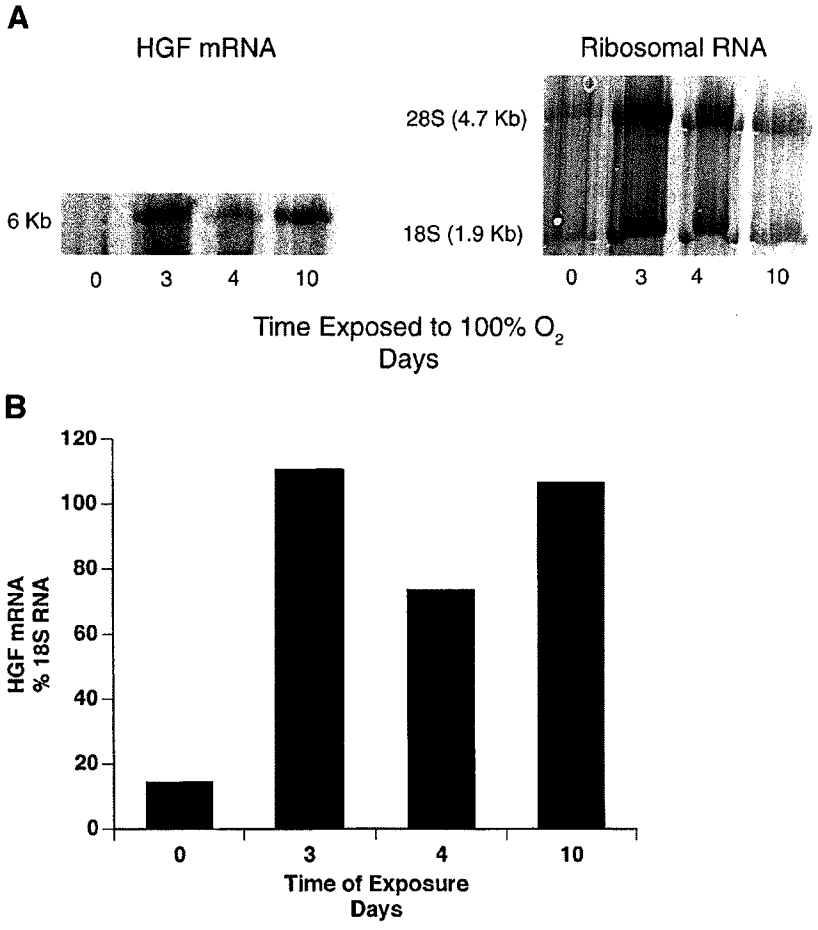

Fig. 1. Northern blot of 50 μg of total RNA obtained from lung tissue of adult rats placed in exposure chamber and exposed to 100% O_2 for times indicated. The rats were then removed from the chamber and immediately killed, and the lungs used to prepare RNA. Total RNA was detected by ethidium bromide staining. *A*: Northern blot with a 1.4-kb cDNA probe of rat hepatocyte growth factor (HGF) and corresponding rRNAs stained with ethidium bromide. Each lane contains RNA from 1 animal. *B*: quantitation by densitometry of ratio of mRNA to rRNA. HGF mRNA was not prominent in tissue from normal adult but was induced in lungs of animals breathing 100% O_2.

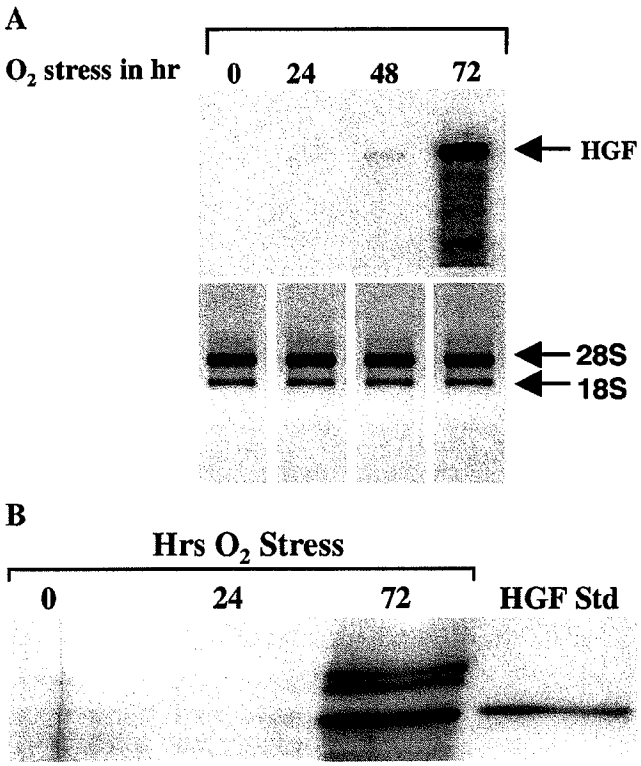

Fig. 2. HGF mRNA and protein in whole lung tissue from rats exposed to 100% O_2 for times indicated. *A*: RNase protection assay (RPA) with 10 μg of total RNA obtained from lung tissue of rats. Ethidium bromide staining of rRNA shows total RNA loaded in each lane. *B*: Western blots of 50 μg of total lung tissue protein loaded in each lane. HGF standard (Std), human HGF. Antibody is a monoclonal anti-human HGF directed to an epitope on α-chain (molecular mass 60 kDa). Larger peptides (molecular mass 90–95 kDa) are likely to be unprocessed prepro- and proHGF isoforms. Each lane contains RNA or protein obtained from lung tissue of 1 animal. Data are representative of 2 independent experiments.

Activation of p42/p44 mitogen-activated PK. Cells seeded in 60-mm tissue culture dishes were treated with HGF (10 ng/ml) for various time periods. The cells were washed twice with PBS and lysed in 200 μl of Tris·HCl buffer (pH 7.4) containing 25 mM Tris, 1% igepal, 150 mM NaCl, 50 mM NaF, 200 μM SOV, and 1 mM phenylmethylsulfonyl fluoride. The lysates were probe-sonicated for 5 s and centrifuged at 14,000 rpm for 10 min to remove the cell debris. Lysate protein was separated on 10% SDS-polyacrylamide, and the gels were probed with anti-phospho-specific p42/p44 mitogen-activated PK (MAPK; New England Biolabs) used at a 1:1,000 dilution in Tris-buffered saline containing 5% BSA and 0.2% Tween 20. Equal protein loading in the gels was confirmed by blotting the membranes with anti-p42/p44 MAPK. The proteins of interest were detected by an enhanced chemiluminescence kit (Amersham). Anti-rabbit IgG-horseradish peroxidase secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:5,000 dilution in the same buffer.

Materials. Rat HGF, as previously described (41), was obtained by recombinant technology by Dr. T. Nakamura, Division of Biochemistry, Osaka University Medical School, Osaka, Japan. Polyclonal antiserum to this recombinant rat HGF was produced in rabbits by conventional methods. Other materials were obtained from commercial suppliers as noted in the text.

Statistical analysis. To compare experiments, specific activities (in cpm/μg PC for metabolic experiments) or enzyme activity units (activity/μg protein) are expressed as a ratio of HGF to control values. These data were tested for significance with a one-factor ANOVA where time and treatment were a combined grouping. Individual means for each group and time were compared with Fisher's protected least squares difference test, and significance was accepted for $P \leq 0.05$. We also tested all data at each time point using an unpaired *t*-test, selecting for groups that differed from a population mean of 1 (no difference between specific activities of HGF and control groups). The results were essentially identical to those obtained with the ANOVA and are not presented.

RESULTS

HGF mRNA and protein are increased in lung tissue and fibroblasts exposed to oxidant stress. HGF may be transcribed in alternative forms (6). The principal transcript is ~6 kb; an alternative transcript of ~2.4 kb, which codes for a truncated form of HGF, competes for membrane receptors and is an inhibitor. The results of a Northern blot from four animals exposed to varying periods of 100% oxygen are shown in Fig. 1. HGF mRNA was very low in the normal animal but markedly increased with exposure to high oxygen. Only the larger transcript was found in either the normal animal or those exposed to 100% oxygen. The smaller (6) transcript was not detected (data not shown).

After determining the size of the HGF transcript, we used RPA to quantify changes in steady-state levels of HGF mRNA in RNA extracted from lung tissue. The results from one of two experiments are shown in Fig.

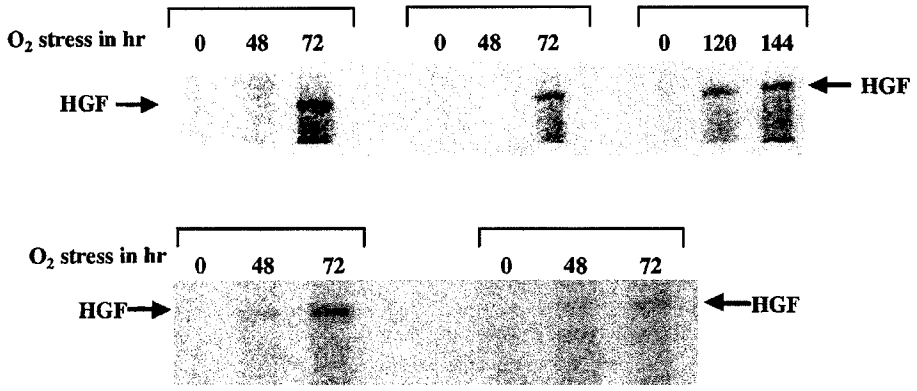

Fig. 3. HGF mRNA from lung fibroblasts obtained from rats exposed to 100% O_2 for times shown. mRNAs were quantified by RPA as indicated in Fig. 2 and described in METHODS. Each lane contains RNA from 1 animal. Data are from 5 separate experiments.

2*A*. HGF mRNA was not detected by RPA in 10 μg of total RNA of nonexposed animals but was evident after 48 h of exposure to 100% oxygen and markedly increased after 72 h. Using quantitative densitometry, we estimate that HGF mRNA increased by >10-fold. The time course of HGF protein was identical to that of the mRNA and is shown in Fig. 2*B*.

We hypothesized that fibroblasts may be a particularly important source of HGF acting on type II cells. Therefore, we assayed steady-state HGF mRNA in fibroblasts isolated from animals exposed to 100% oxygen for 2–6 days and then maintained in culture through two passages. Shown in Fig. 3 are the results obtained from five experiments. HGF mRNA was not detected in normal animals but became notable after 3 days of exposure (based on four animals that were examined) and was still prominent at 5–6 days (based on two animals). The results suggest that the source of the increased HGF after injury may be, in part, fibroblasts.

Tumor necrosis factor-α and interleukin-1β induce the transcription of HGF in fibroblasts. We sought possible mechanisms for the increase in HGF found in animals with lung injury induced with 100% oxygen. Cytokines are produced early in the inflammatory phase of lung injury, and cytokines have been reported to induce HGF in stabilized fetal cell lines (40). We studied, therefore, whether cytokines would be able to induce HGF mRNA in lung fibroblasts of normal, uninjured adult animals.

Lung fibroblasts were isolated and placed in culture for two passages. About 10 million cells were given varying concentrations of either tumor necrosis factor (TNF)-α or interleukin (IL)-1β for 24 h, the cells were then harvested, and total RNA was isolated. HGF mRNA was quantified by RPA. The results of one experiment are shown in Fig. 4 and are representative of those from two independent experiments. HGF mRNA was undetectable in control fibroblasts. However, in cells exposed to either 1 ng/ml of TNF-α or 1 ng/ml of IL-1β, HGF mRNA was abundant. Using densitometry of the gels to estimate the changes induced by the cytokines, we found that 1 ng/ml of TNF-α increased HGF mRNA by >300-fold (320 ± 83, mean ± range of 2 experiments) and 1 ng/ml of IL-1β increased HGF mRNA by >3-fold (3.25 ± 0.95).

Changes in surfactant metabolism induced by HGF. The time course of the changes seen in cell and medium pools from one experiment is shown in Fig. 5. Because HGF activates signaling pathways by tyrosine phosphorylation of c-met (24), we included 20 μM SOV (39) in all wells, both control and HGF treated. Cells accumulated radioactivity in PC pools throughout the entire course of the 45- to 48-h experiments, although the rates of accumulation plateaued after 24 h. In contrast, specific activities in medium increased with time. The data suggest sustained metabolic viability throughout the course of the experiment, with a probable tendency toward equilibration between new synthesis and reuptake in the cell pool. The mean changes induced by HGF from all experiments pooled and tested for significance are seen in Fig. 6. HGF combined with SOV decreased specific activities in cells to 60–75% of control values at 5–48 h ($P \leq 0.05$). In medium, the

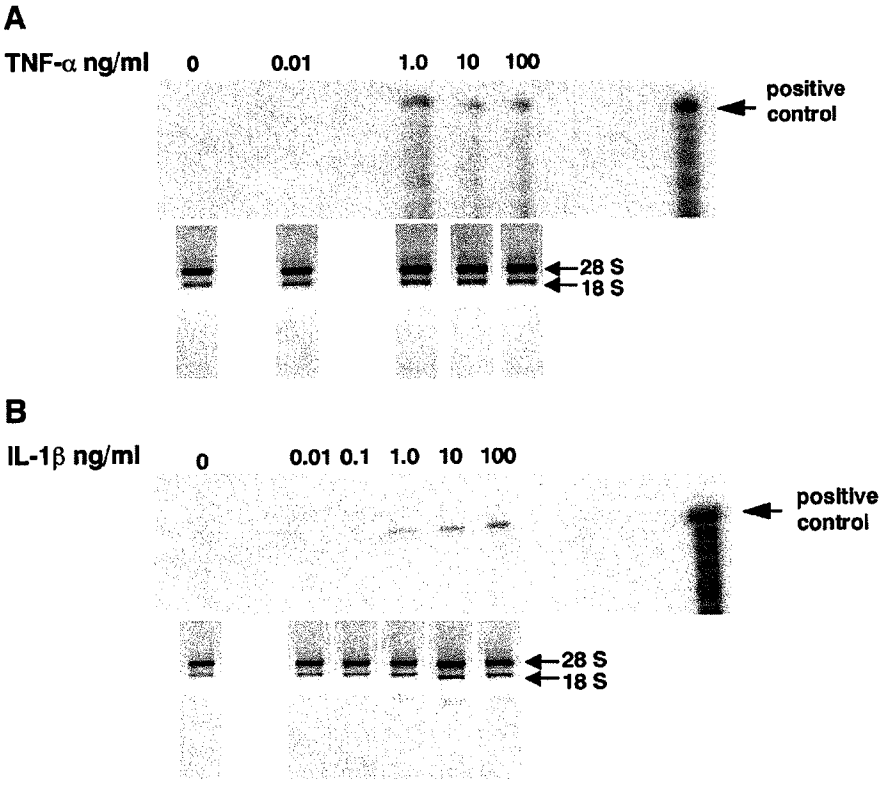

Fig. 4. HGF mRNA in lung fibroblasts from normal rats that had been presented with varying concentrations of either tumor necrosis factor (TNF)-α (*A*) or interleukin (IL)-1β (*B*). Fibroblasts were obtained from rats breathing room air and were passaged twice before experiment. They were then given indicated concentrations of TNF-α or IL-1β for 48 h. HGF mRNA was determined by RPA. *A* and *B*, *top*: protected fragments of HGF mRNA. *A* and *B*, *bottom*: ethidium bromide staining of total RNA. Data are representative of 2 independent experiments, each using cells from 1 animal.

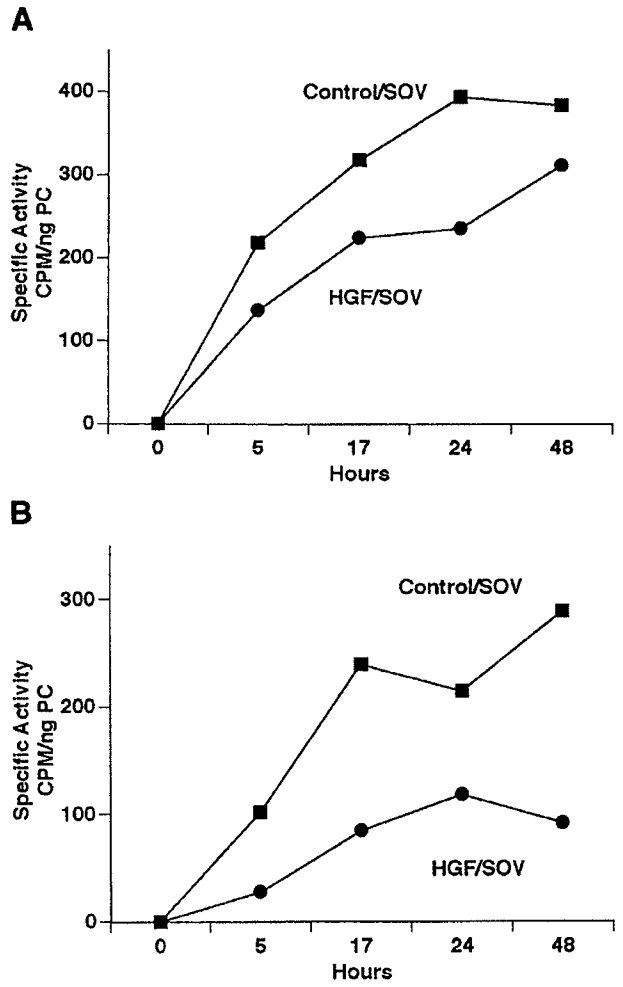

Fig. 5. Effects of 5 ng/ml of rat HGF together with 20 μM sodium orthovanadate (SOV) on specific activities of phosphatidylcholine (PC) in cultures of rat type II cells. Type II cells were isolated from normal adult rats of ~175–200 g body wt and placed on uncoated Millipore cell culture inserts overnight in DMEM containing 10^{-4} M hydrocortisone, 5×10^{-4} M cAMP, 1 μl/ml of insulin-transferrin-selenium, and 6 μM arginine-glycine-aspartic acid-serine without FCS. The following day, medium was replaced with that containing 5 ng/ml of HGF together with [^{32}P]SOV. Cells and media were recovered at times shown, lipids were extracted, and specific activities in PC were determined. *A*: cell lysates. *B*: medium. Data are means of 3–6 wells at each time point taken from 1 experiment. CPM, counts/min.

specific activity at 5 h was reduced but was not significant. At all other times, however, the inhibition of medium specific activity was greater than the inhibition in cells. Specific activities of medium from HGF plus SOV-treated cells were generally reduced to 40–80% of control values in the time period between 17 and 48 h and in occasional individual experiments, even less. When we compared control cells with and without SOV, we found that SOV by itself did not markedly affect the synthesis or secretion of phospholipids (data not shown).

HGF used alone also reduces cell and medium specific activities, but the inhibition is less than when SOV is included (Fig. 7). Cell specific activity at 5 h was ~65% of control specific activity ($P \leq 0.05$) but was reversed at 17–48 h to ~85–90% of control specific activity ($P \geq 0.1$). In medium, there were no significant changes at 5 h. From 17 to 48 h, however, HGF induced decreases in medium specific activities, with significant reductions from 17 to 48 h, which varied from 65 to 85% of control value.

Amounts and composition of surfactant PCs are unchanged by HGF. Neither HGF nor HGF-SOV increased the amount of cellular PC, concomitant with a possible stimulation of cell division, but this would not be expected because we used nonsynchronized cell cultures, and none contained serum, which is necessary for cell division (27). Medium pools in some experiments were reduced (in one experiment, to ~35% of the corresponding control pools), but the results were varied and were not significant in the averaged experiments.

The compositions of the cell and medium PC pools were routinely measured. Neither was affected by HGF. Within the limits of our detection, the medium was composed of 80–90% disaturated PC and the cells >60%.

HGF inhibits the activity of CT. CT is the rate-limiting enzyme in the de novo synthesis of PC (29), and we evaluated the effects of HGF on its activity.

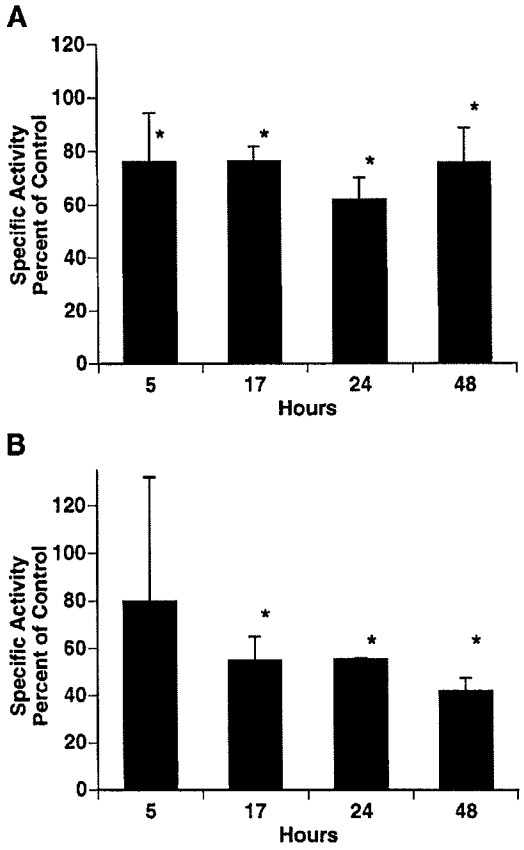

Fig. 6. Changes in specific activity of PC induced by 5 ng/ml of HGF with 20 μM SOV. *A*: cell lysates. *B*: medium. Values are means ± SE from 3 experiments. * $P \leq 0.05$, compared with time-matched control value.

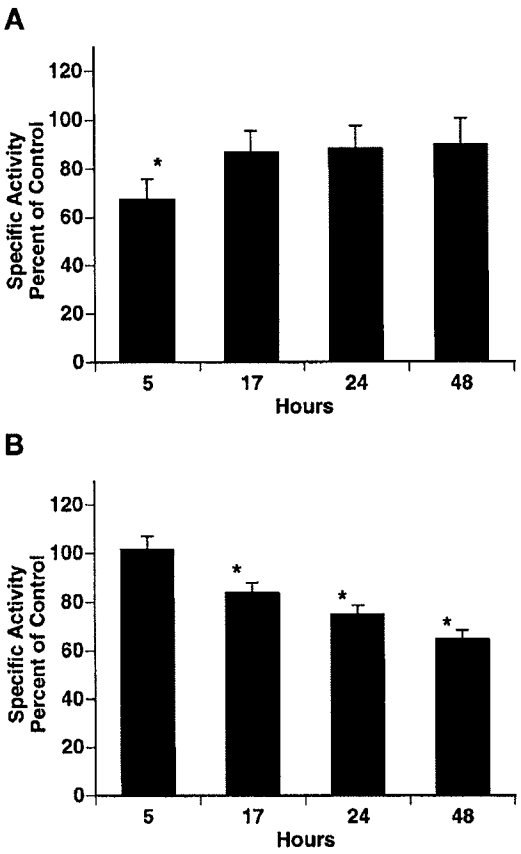

Fig. 7. Changes in cell specific activity induced by 5 ng/ml of HGF without SOV. *A*: cell lysates. *B*: medium. Values are means ± SE from 3 experiments. * $P \leq 0.05$, compared with time-matched control value.

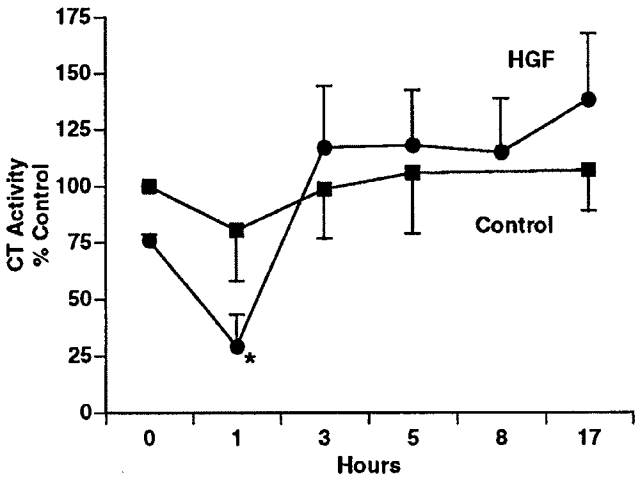

Fig. 8. Changes in CTP: choline-phosphate cytidylyltransferase (CT; EC 2.7.7.15) activity in NCI-H441 cells treated with 5 ng/ml of HGF. Cells were placed in cell culture medium without FCS for 1 h before experiment and were then given HGF or vehicle buffer (control). Cells were harvested at times shown, and CT activity was measured in membrane fraction. Data are expressed as percent of activity in control cells harvested at 0 h. Values are means ± SE from 3 experiments. * $P \leq 0.05$, compared with time-matched control value.

Because type II cells are difficult to obtain in large quantities, we used H441 cells to develop a time course of the effects of HGF on the activity of CT (Fig. 8). H441 cells are frequently used for the study of surfactant because they synthesize several of the proteins of surfactant (47) and demonstrate other characteristics of a surfactant-producing cell (26). The results show that HGF inhibits membrane-bound CT activity between 0 and 1 h. The effects are relatively rapid because the 0-h data, which more accurately reflect the ~5 min required for chilling the cells to ~5°C and processing, show a reduced (but not significant) activity in all samples. The inhibition was reversed by 3 h. This time course for the change in CT activity, therefore, is compatible with the observed inhibition in cellular synthesis of PC found after 5 h of HGF but reversed after 17 h.

Two mechanisms for the regulation of CT have been proposed: *1*) increases in phosphorylation, which reduce the binding of CT to lipid membranes, thereby decreasing activity (3); and *2*) changes in the content of activating lipids, namely DAG and fatty acids (20, 42). Changes in DAG content may, in turn, depend on the activation of PKA (20), which phosphorylates acetyl-CoA carboxylase and inhibits its activity (19), thereby reducing fatty acid synthesis and DAG content.

PKA activity is stimulated by HGF. We measured the effects of HGF on PKA activity in primary cultures of type II cells. Cells were treated for 1 h with 5 ng/ml HGF. Control cells were treated similarly, except that HGF was omitted. The results from two experiments showed that HGF increased PKA activity by 40 and 70%. These effects on PKA are consistent with those reported by Grumbles et al. (17), who found that HGF

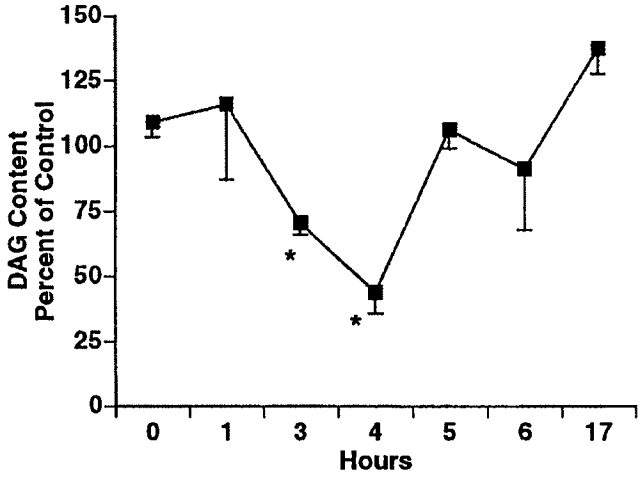

Fig. 9. Diacylglycerol (DAG) contents in H441 cells treated with 5 ng/ml of HGF. H441 cells were plated in 10% FCS-McCoy's 5A medium; 1 h before reagents were added, medium was changed to McCoy's 5A without FCS containing [^{14}C]palmitic acid. At 0 h, HGF or solvent was added to cells, and cells were harvested by scraping at times shown. Cellular lipids were extracted, and 1,2-DAG was isolated by TLC on plates of silica gel H with solvent system ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2 vol/vol), followed by ethyl ether-hexane (6:94) (47). 1,2-DAG was scraped, and content was determined by scintillation counting. Data are percent of time-matched control cells. Values are means ± SE. * $P \leq 0.05$, compared with time-matched control value.

induced a modest but statistically significant increase in PKA activity in bone cells from rat tibia.

DAG content is reduced in cells treated with HGF but not at times consistent with CT inhibition. H441 cells were treated with HGF for times varying from 1 to 17 h, and DAG content was measured (Fig. 9). DAG content was reduced by ~50% at 3 and 4 h, consistent with changes in PKA activity at 1 h. However, there were no detectable changes in DAG content at 1 h. This mechanism, therefore, cannot explain the rapid decrease in CT activity at the 1-h and 0-h time points.

HGF rapidly activates p42/p44 MAPK and effects of HGF on CT are reversed by an inhibitor of proline-dependent kinases. CT contains numerous serines clustered near the carboxy terminus (21), and seven of these have consensus sequences for putative substrates of proline-directed kinases. HGF has been reported to activate p42/p44 MAPK in hepatocytes (1, 15) and H441 cells (8), providing a possible mechanism for the rapid inactivation of CT by HGF. We confirmed that HGF activated p42/p44 MAPK under the conditions in which we conducted our experiments, as shown in Fig. 10. HGF phosphorylates p42/p44 MAPK in a time-dependent manner, suggesting a corresponding time course of kinase activity. Abundance of phosphorylated MAPK peaked by 10 min to 180 ± 19% (SE) of control level (n = 3 experiments) and returned to about control levels at 1 h. Total p42/p44 MAPK was not changed.

If p42/p44 MAPK phosphorylates CT and subsequently reduces its activity, we would expect that an inhibitor of this MAPK would mitigate this effect. We used 40 μM olomoucine, a purine derivative, because, at low concentration, it is a relatively specific inhibitor of proline-dependent kinases, including p42/p44 MAPK (IC_{50} for p44 MAPK is 25 μM), but it does not affect other types of kinases, such as PKC (44). Olomoucine has been used previously in human keratinocytes to demonstrate that the phosphorylation of CT, and reduction in its activity, may involve a proline-dependent kinase (46). Isoolomoucine, which is inactive at this concentration, was used as a negative control. The results are shown in Fig. 11. Olomoucine blocks the inhibition of CT activity by HGF at 1 h ($P \leq 0.01$),

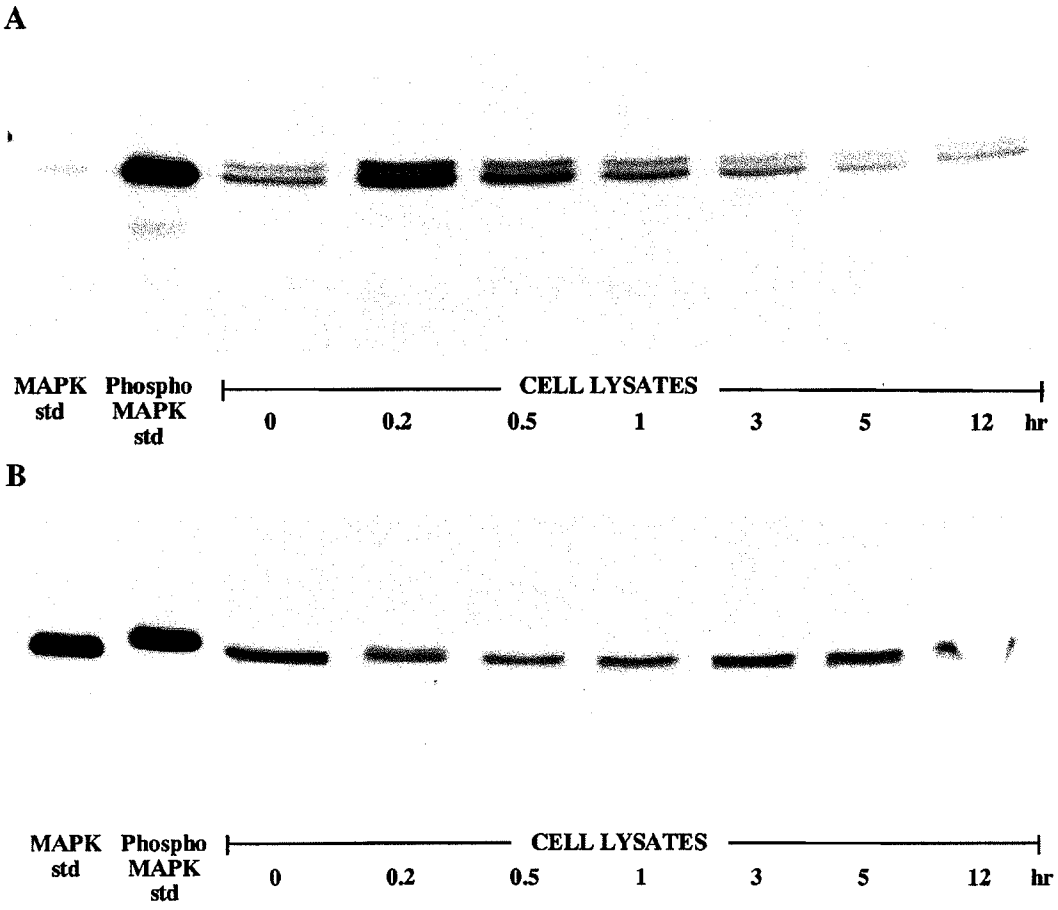

Fig. 10. Changes in p42/p44 mitogen-activated protein kinase (MAPK) induced by HGF. Cells were washed, scraped, and processed for SDS-PAGE and Western blots. Blots were developed with primary antibodies to either phospho-specific p42/p44 MAPK (*A*) or nonphosphorylated MAPK (*B*), both in dilutions of 1:1,000. *Lane 1*, nonphosphorylated MAPK control protein; *lane 2*, phosphorylated (Phospho) MAPK control protein; *lanes 3–9*, 25 μg of cellular protein from cells exposed to 10 ng/ml HGF for times indicated. Results are representative of 3 experiments.

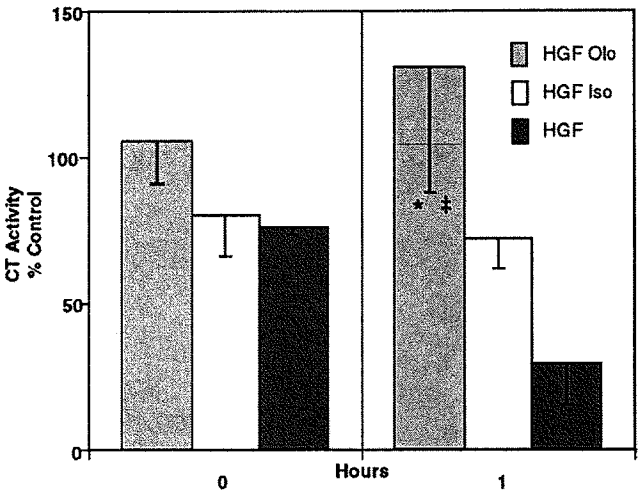

Fig. 11. Effects of olomoucine (Olo) and isoolomoucine (Iso) on changes in CT activity induced by HGF. H441 cells were treated with 5 ng/ml of HGF for 0 and 1 h as described in Fig. 8. Wells also contained either 40 μM Olo (an inhibitor of p42/p44 MAPK) or 40 μM Iso (negative control). Data are expressed as percent of untreated control (100% data not shown). Cells given HGF plus Olo or HGF plus Iso were compared with those obtained with HGF alone, taken from Fig. 8. Olo prevented HGF-induced inhibition of CT, whereas Iso was less effective. Values are means ± SE. ‡ $P \leq 0.01$ for HGF plus Olo vs. HGF. * $P \leq 0.05$ for HGF plus Olo vs. HGF plus Iso. HGF plus Iso was not significant when compared with HGF ($P \geq 0.10$).

whereas isoolomoucine was less effective, and its effects were not significant.

HGF does not inhibit PKC activity. In a now classic and oft quoted paper, Sano et al. (36) demonstrated that the secretion of surfactant PC in isolated type II cells is stimulated by agonists that stimulate PKC. In the past 15 years, several other investigators have identified other purported secretagogues of PC, and several of these, when investigated, appear to converge on PKC early in their respective signaling pathways. [See, for instance, work on low- and high-density lipoproteins (30), ATP (7, 16, 31), and endothelin-1 (37).] Thus it would appear that PKC has an important role in the signaling pathways used by several secretagogues of surfactant PC and that it occupies an early upstream position in those pathways. To look for a direct connection between changes in PKC activity and the inhibition of PC secretion by HGF, we assayed PKC activity in the membrane fraction of lysates of H441 cells at times varying from 17 to 41 h. At these times, we found a significant reduction in the specific activity of PC in the medium. Membrane-associated PKC activity in these cells (expressed as a percentage of control cells) was as follows: 17 h, 93.7 ± 3.8%; 24 h, 105.3 ± 9.5%; 41 h, 87.7 ± 3.3%. HGF does not significantly reduce membrane-associated PKC activity in the time period when secretion is inhibited.

DISCUSSION

These results may have relevance to the pathophysiological changes that occur with chronic lung injury. Well documented in this condition (as in injuries in other tissues) is an early surge of inflammatory cytokines, prominently TNF-α and IL-1β, followed by increased amounts of growth factors (11, 22) accompanying an increased proliferation of epithelial cells and fibroblasts. Our data are consistent with these in vivo observations because they show increased levels of an alveolar epithelial mitogen, HGF, and because they demonstrate that this induction may be through cytokine stimulation. Our data also help explain the reduction in pulmonary surfactant also seen in ARDS, a seemingly paradoxical finding in light of the increased numbers of type II cells (10).

We used H441 cells to explore possible biochemical pathways that might be interrupted by HGF. These cells have been widely used to study surfactant metabolism. Because these experiments required very large numbers of cells, their utilization in this context is very attractive. Based on results found in these cells, we hypothesize that the effects of HGF on surfactant synthesis may be due to a reduction in CT activity either via its phosphorylation by a MAPK or through the MAPK-dependent phosphorylation of an intermediate that ultimately impacts CT activity. Consistent with this hypothesis, the effects of HGF on CT are reversed by an inhibitor of proline-dependent kinases, which would include all three classes of MAPK. We have not attempted, however, to discern which of the proline-dependent kinases are actually involved. Such studies would probably involve multiple inhibitors and the use of dominant negatives in transfected cells and are beyond the scope of our current experiments. Clearly, the relevant kinase may be one other than p42/p44 MAPK. The effects of HGF on surfactant are also increased when protein tyrosine phosphatases are inhibited by SOV, presumably in part by potentiating tyrosine phosphorylation on the HGF receptor (24), although other intermediaries in the signal transduction pathway would also be affected. An alternative mechanism proposed for the regulation of CT activity is through changes in the content of activating lipids, namely DAG and fatty acids (20). We found a decrease in DAG content induced by HGF after 3–4 h, but this change cannot explain the decrease in CT activity at 1 h. We also observed an increase in PKA activity after 1 h, and CT is a substrate for PKA phosphorylation in vitro (29, 35). It is uncertain whether this mechanism has relevance in intact cells (20).

We have been unable to provide a mechanistic explanation for the inhibition of secretion that is observed at times >17 h. This is a time span likely to involve mRNA transcription and new protein synthesis, but such proteins have not been identified. Other papers also indicate that secretion is inhibited only after long exposure to a cytokine or growth factor; witness the effects on surfactant found for TNF-α (4, 47) and transforming growth factor-β (45). PKC is a known secretagogue of surfactant PC, and we reasoned that this long time course might involve the synthesis of a regulator protein that inactivates PKC just before the observed inhibition of secretion. This was not found to be the case. PKC activity was not reduced between 17 and 41 h, implying that PKC is not involved in this regulation. An alternative explanation (and one less

likely in our opinion) is that the inhibition is through a PKC isoform in relatively low abundance compared with other PKC types, and the changes in it are not detected by a relatively nonspecific assay of PKC activity.

This is, to our knowledge, the first report that HGF inhibits lung PC synthesis and secretion, possibly, in part, through the inhibition of CT. The effects of HGF in ARDS may extend beyond localized actions in the lung. Lung cells not only synthesize increased amounts of HGF in localized lung injury, but they also appear to release HGF into the circulation as they respond to humoral substances released by extrapulmonary tissues undergoing acute inflammatory injury (48, 49). Thus HGF produced by the lung in response to localized injury may result in wider effects reflected in systemic changes in PC metabolism.

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Vascular endothelial growth factor synthesis in the acute phase of experimental and clinical lung injury

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Vascular endothelial growth factor synthesis in the acute phase of experimental and clinical lung injury. B. Maitre, S. Boussat, D. Jean, M. Gouge, L. Brochard, B. Housset, S. Adnot, C. Delclaux. ©ERS Journals Ltd 2001.

ABSTRACT: Vascular endothelial growth factor (VEGF) is a potent angiogenic and endothelial survival factor, which is abundantly expressed in the normal lung. Conceivably, VEGF may be released by numerous cell types found around the airspaces, including alveolar type 2 cells, alveolar macrophages, and polymorphonuclear neutrophils.

Using a bacteria-induced lung injury model in rats, VEGF expression in lung was investigated. Both VEGF protein and VEGF messenger ribonucleic acid (mRNA), 4 and 24 h after bacterial challenge (*Pseudomonas aeruginosa*), were decreased compared with sham rats.

VEGF protein was also investigated in bronchoalveolar lavage (BAL) from patients studied within 7 days of acute respiratory distress syndrome (ARDS) onset and in patients without ARDS. VEGF protein levels in BAL were decreased in patients with ARDS *versus* those without (14.3 ± 11.1 pg·mL^{-1} *versus* 76.8 ± 51.1 pg·mL^{-1}, $p = 0.03$).

In aggregate, these findings show that the initial phase of acute lung injury is associated with a decrease in vascular endothelial growth factor in the lung. This downregulation may represent a protective mechanism aimed at limiting endothelial permeability, and may participate in the decrease in capillary number that is observed during early acute respiratory distress syndrome.

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The acute respiratory distress syndrome (ARDS) is a clinical and pathophysiological entity characterized by acute, diffuse injury to the alveolar-capillary wall responsible for respiratory failure. Increases in capillary and alveolar permeability result in interstitial and alveolar oedema, respectively. The endothelial permeability increase is thought to involve both neutrophil-dependent and neutrophil-independent mechanisms [1]. Among factors that can increase endothelial permeability, the vascular endothelial growth factor (VEGF) family has not yet been investigated in ARDS. Three different forms of VEGF are currently known: VEGF-A (or VEGF), VEGF-B and VEGF-C, each form having a different affinity for different membrane receptors: flt-1(or type 1 receptor), fl-k1(or kinase insert domain-containing receptor (KDR) or type 2 receptor), type 3 receptor and neuropilin-1. VEGF is a highly conserved, dimeric, heparin-binding glycoprotein (molecular weight 46 kDa). At least four different VEGF transcripts resulting from alternate splicing of a single gene have been described in human cells [2]. VEGF 121 and 165 are secreted in soluble form, whereas VEGF 189 and 206 remain cell-surface associated or are primarily deposited in the extracellular matrix. VEGF seems to specifically affect endothelial cell survival, growth and permeability through binding to the high affinity transmembrane tyrosine kinase receptor flk-1, predominantly located on endothelial cells. In the lung, VEGF may be expressed primarily by epithelial cells and macrophages [3, 4]. Recently, VEGF has also been shown to be synthesized and stored by human polymorphonuclear neutrophils [5, 6]. Although increased capillary wall permeability is the hallmark of ARDS, a decrease in capillary number and volume sets in rapidly, and is a hallmark of subacute and chronic stages of ARDS [7]. A fibroproliferative process characterized by collagen synthesis in the lung, as reflected by elevated levels of procollagen III in pulmonary oedema fluid, begins within the first 24 h of acute lung injury, concurrently with the acute phase of increased endothelial and epithelial permeability to proteins [8]. Macroscopically, intra-alveolar fibroproliferation (including capillary proliferation) is visible within 7 days [9], indicating that the process probably starts within the first few days of injury.

Since VEGF, besides its increasing endothelial permeability capability, has recently been described as a major survival factor for endothelial cells, the aim of the present study was to evaluate its evolution at the initial phase of ARDS. To explore the potential function of VEGF in acute lung injury, VEGF was evaluated in a previously described animal model of severe acute lung injury [10]. The VEGF concentration in bronchoalveolar lavage (BAL) fluid from patients with severe ARDS at the initial phase of

respiratory failure (within the first 7 days) was also evaluated comparatively with patients who were receiving mechanical ventilation, but did not have respiratory failure.

Material and methods

Rat model of acute lung injury

Male Sprague Dawley rats weighing ~250 g were used. The rats were housed in air-filtered, temperature-controlled units, and were allowed free access to food and water.

Alveolar instillation of a *Pseudomonas aeruginosa* inoculum was performed as previously described [10, 11]. Briefly, the rats were anaesthetized with halothane administration, and the trachea was exposed. A *P. aeruginosa* inoculum (strain 011.4; 8×10^9 *P. aeruginosa*·mL^{-1}) in sterile physiological saline was instilled into the trachea using a 25-gauge needle, in a dose of 0.5 mL·kg^{-1}. Sham-treated rats were instilled with 0.5 mL·kg^{-1} of sterile physiological saline. Studies were done 4 h, 24 h and 5 days after tracheal instillation of bacteria or saline. The rats were exsanguinated, and the lung and trachea were removed *en bloc*. The right lung was snap-frozen in liquid nitrogen for ribonucleic acid (RNA) and protein extraction. To preserve tissue architecture, OCT (Tissuetek, Paris, France) diluted 50% in phosphate buffered saline was instilled *in situ* into the left lung, which was then snap-frozen in liquid nitrogen for histologic examination.

Patient selection and sample collection

Mechanically-ventilated patients were prospectively studied in the medical intensive care unit of the Henri Mondor Hospital, Créteil, France. Patients scheduled for imminent BAL to evaluate suspected ventilator-associated pneumonia were eligible for the study. To avoid potential confounding due to local cell function changes induced by nosocomial pneumonia, patients with nosocomial pneumonia were excluded, based on the results of quantitative culture of BAL fluid and protected plugged catheter [12].

Patients were divided into two groups based on whether or not they had ARDS, defined as bilateral infiltrates on a frontal chest radiograph with a arterial oxygen tension/inspiratory oxygen fraction ($Pa,O_2/Fi,O_2$) ratio <150 mmHg, regardless of positive end-expiratory pressure level, and a pulmonary wedge pressure <18 mmHg. Non-ARDS (control) patients were required to have a $Pa,O_2/Fi,O_2$ ratio >300 mmHg under mechanical ventilation. The study was approved by the Ethics Committee of the hospital. Written informed consent was obtained from the next of kin.

BAL was performed as previously described [12]. Briefly, three 50 mL aliquots of sterile, pyrogen-free, 0.9% NaCl were instilled and recovered using gentle suction. The fluid recovered after the first 50 mL instillation was discarded. BAL fluid was filtered through moistened coarse gauze to remove mucus. At the same time, 20 mL of arterial blood was drawn. A small BAL aliquot was used for a total cell count and cytocentrifuge preparations. The total cell count was done manually using a haematocytometer. Cell smears were stained with standard May-Grünwald Giemsa. Differential cell counts were performed by counting 200 cells. BAL was centrifuged at $300 \times g$ for 7 min immediately after collection. BAL fluid was aliquoted and frozen at -80°C until use.

Urea determination

Urea concentration was determined by spectrophotometric absorbance at 600 nm using the Berthelot reaction (Diagnostic Kit, Boehringer, Mannheim, Germany). The volume of epithelial lining fluid (ELF) recovered by BAL was estimated using the following formula with urea as a marker of dilution: ELF volume (L) = (total amount of urea in recovered BAL fluid (mmol))/(plasma urea concentration (mmol·L^{-1})) [13].

Biological assay

Enzyme-linked immunosorbent assay for vascular endothelial growth factor and transforming growth factor-β1. VEGF and transforming growth factor-β1 (TGF-β1) protein levels were quantified in BAL supernatant and serum using a sandwich enzymetric assay with specific antibodies, as recommended by the manufacturer (R and D System, Minneapolis, MN, USA). Activation for TGF-β1 detection was made using acidification and neutralization procedures. A 200 μL sample was incubated with 50 μL of assay diluent for 2 h at room temperature in a 96-well plate, coated with a monoclonal antibody. After three washings, a polyclonal antibody-horseradish peroxidase conjugate was added. Incubation was for 2 h at room temperature. After addition of a colour reagent, absorbance was measured at 450 nm using a Thermo-Max microplate reader (Dynatec Laboratories, El Paso, TX, USA). For standardization, serial dilutions of recombinant human growth factor were assayed during the same run.

Immunoblotting of vascular endothelial growth factor isoforms. Tissue lysates were prepared from rat lung in lysis buffer containing 20 mM hydroxyethyl piperazine ethane sulphonic acid (HEPES), 0.5 mM ethylene glycol tetra-acetic acid (EGTA), 1 mM DTT and 0.32 M sucrose, pH 7.4. Protein content was determined using the method of Lowry as modified for the Detergent Compatible protein assay (Bio-Rad, Richmond, CA, USA). Samples (30 μg·lane^{-1}) were subjected to polyacrylamide gel electrophoresis as described by HOSSENLOPP *et al.* [14]. Separated peptides were transferred electrophoretically to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA), which were incubated overnight with a blocking solution containing Tris-buffered saline with 0.05% Tween 20 (TBS-T) and 3% bovine serum albumin (BSA). VEGF proteins were detected by incubating the membrane with a polyclonal antihuman VEGF antibody (Santa Cruz Laboratories, Santa Cruz,

CA, USA). The blots were then washed and incubated with peroxidase-conjugated goat antirabbit immunoglobulin-G (IgG) (Dako A/S Co., Glostrup, Denmark). Immune complexes were visualized using the enhance chemiluminescence detection system (Amersham Corp., Buckinghamshire, UK), and membranes were exposed to autoradiography film (Amersham Corp.).

Analysis of messenger ribonucleic acid expression by reverse transcriptase-polymerase chain reaction amplification and Northern blotting. Total RNA was extracted from the lungs according to an improvement to the single-step RNA isolation method developed by CHOMCZYNSKI and SACCHI [15]. Total RNA was quantified at 260 or 280 nm, and sample integrity was checked using 1.5% agarose gel electrophoresis. Total RNAs (2 μg) were converted to complementary deoxyribonucleic acids (cDNAs) using 5 units of Maloney murine leukaemia virus (MMLV) (Gibco BRL, Life Technologies, France) and 0.5 μg oligo deoxythymidine (dT) for 1 h at 37°C. Reverse transcriptase (RT)-generated cDNA encoding VEGF-A and β-actin (used as a control of RNA integrity and as an internal standard) were amplified using polymerase chain reaction (PCR). Amplification of 5 μL of cDNA was performed using 0.2 nM sense and antisense VEGF primers and 2.5 units of *Taq* polymerase. The oligonucleotide primer sequences for VEGF were 5′CCATGAACTTTCTGCTCTCTTG3′ (sense) and 5′GGTGAGAGGTCTAGTTCCCGA3′ (antisense). ^{32}Phosphate-labelled deoxycytidine triphosphate (^{32}P-dCTP) was added (0.3 μM for each sample). Samples were amplified for 30 PCR cycles. A 5 μL aliquot from each PCR reaction was subjected to polyacrylamide gel electrophoresis and visualized by autoradiography. Their authenticity was confirmed by direct nucleotide sequencing. All reverse transcriptase-polymerase chain reaction (RT-PCR) studies were performed at least four times, using lung RNA from different animals.

For Northern blotting, 20 μg of RNA were fractionated by electrophoresis on 1% agarose, 2.2 M formaldehyde gels and blotted onto nylon membranes (Stratagene, La Jolla, CA, USA). The blots were prehybridized and hybridized to ^{32}P-labelled probes, washed, and exposed to film. Relative band intensity was quantified by scanning densitometry using the intensity of a 28S ribosomal ribonucleic acid (rRNA) ethidium bromide-stained band as the comparator. The plasmid containing the VEGF165 insert was obtained by subcloning of PCR amplification product, from rat lung cDNA, into a pCRII vector (In Vitrogen, San Diego, CA, USA) and subjected to DNA sequence analysis (Sequenase, US Biochemical Corp., Cleveland, OH, USA). The probe was generated by labelling plasmid inserts with (α-P)dCTP using random oligonucleotide priming (Amersham Corp.).

Statistical analysis

All data are expressed as mean ± SEM. Between-group comparisons were done using nonparametric methods, namely the Mann-Whitney U-test or Spearman rank correlation coefficient as appropriate. Significance was defined as $p < 0.05$.

Results

Vascular endothelial growth factor expression in rat acute lung injury

The 50% 24-h mortality rate in this animal model attests to the severity of the lung injury. Histological examination of surviving rats demonstrated diffuse alveolar damage, with infiltration of polymorphonuclear neutrophils and presence of oedema (fig. 1).

Expression in the rat lungs of three VEGF

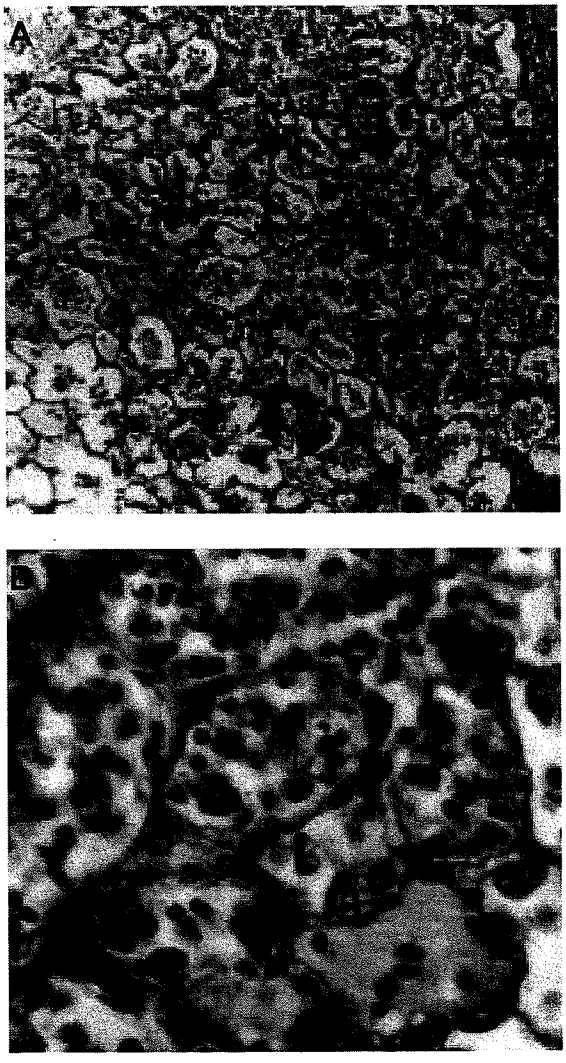

Fig. 1. – Histological section of rat lung: A) original magnification ×200; and B) original magnification ×300, 24 h after intratracheal instillation of *Pseudomonas aeruginosa*. Alveolar spaces were filled with oedema and inflammatory cells.

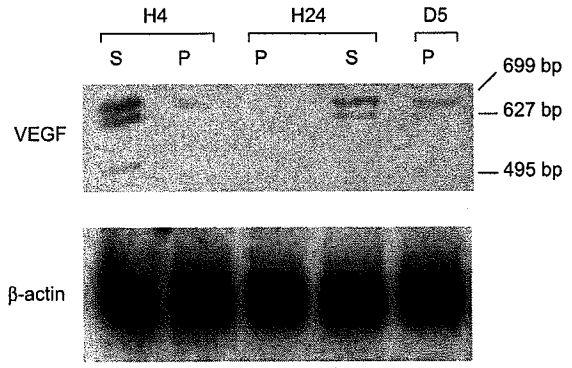

Fig. 2. – Reverse transcriptase-polymerase chain reaction (RT-PCR) of vascular endothelial growth factor (VEGF) messenger ribonucleic acid (mRNA) expression in lung of sham (S) and *Pseudomonas aeruginosa*-instilled rats (PA). Equal amounts of complementary deoxyribonucleic acid (cDNA) were amplified by RT-PCR for VEGF and β-actin using 30 cycles. PCR products were resolved by 5% polyacrylamide gel electrophoresis and were analysed by autoradiography. Three different cDNAs were amplified in rat lung corresponding to VEGF 121, 165 and 189. β-actin was used as a control for mRNA integrity for each sample. bp: base pair.

messenger ribonucleic acid (mRNA) species corresponding to the VEGF 121, 165, and 189 isoforms was evaluated using RT–PCR (fig. 2). No differential expression of VEGF mRNA splice variants were observed during acute lung injury in the model. Northern hybridization of rat lung RNA disclosed a major 3.4 kb transcript and a faint signal at 1.5 kb. As shown in figure 3, VEGF mRNA decreased at 4 hr, and was further decreased at 24 hr. This decrease was reversible: VEGF mRNA levels in survivors five days postinjury were similar to those in sham animals.

Results of immunoblot analysis of VEGF proteins are shown in figure 4. VEGF protein levels were decreased in lung-injured *versus* sham animals at 4 h and 24 h. This effect was also reversible, with VEGF protein returning to baseline within 5 days after the injury.

Clinical parameters

As these results were found in this animal model, an attempt was made to confirm them in patients with ARDS. Nineteen patients were studied and thirteen patients (seven males, six females; mean age, 50±5 yrs) had severe ARDS, with a persistently low $Pa,O_2/FI,O_2$ ratio (158±19 mmHg) at the time of BAL (3±1 days after ARDS onset). The cause of ARDS was direct lung injury in seven cases and indirect lung injury in six. Survival rate was 5/13 (38%). The non-ARDS group included six patients without respiratory injury ($Pa,O_2/FI,O_2$ ratio >300 mmHg) (five males, one female; mean age, 59±5 yrs). None of the control group patients developed ARDS during the remainder of their intensive care unit stay.

Bronchoalveolar lavage parameters

ARDS patients had higher ELF volumes than non-ARDS patients: 9.9±2.5 mL·100 mL^{-1} BAL fluid

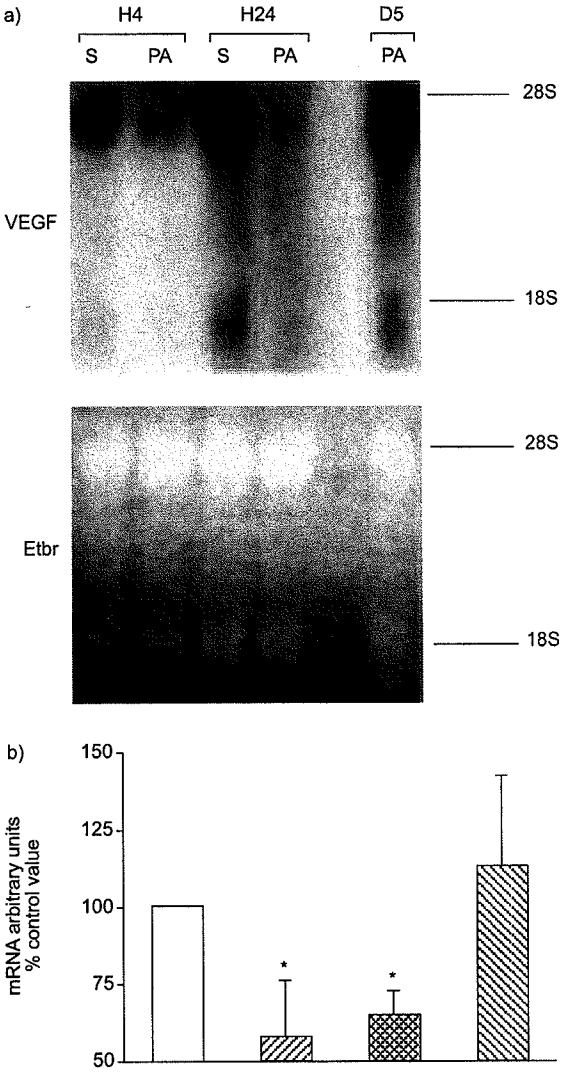

Fig. 3. – Expression of vascular endothelial growth factor messenger ribonucleic acid (mRNA) in rat lung from sham (S) and *Pseudomonas aeruginosa*-instilled rats (PA). a) Autoradiogram of the hybridization signals with the ethidium bromide (Etbr) staining of the gel on the bottom; and b) histogram showing quantitative representation of vascular endothelial growth factor (VEGF) hybridization obtained from laser densitometric analysis of the exposed films (four independent experiments). Total RNA was extracted from lung of sham (□) and PA animals 4 h (H4, ▨), 24 h (H24, ▩) and 5 days (D5, ▧) after instillation. Expression of VEGF was analysed by Northern blotting using specific human probes, as indicated in Material and methods. *: $p<0.05$ *versus* sham condition at the same time (H4 and H24).

versus 1.9±0.6 mL·100 mL^{-1}, respectively ($p<0.005$). Total BAL fluid cell count was not significantly increased in mechanically ventilated patients with and without ARDS (430±102×10^3·mL^{-1} for ARDS *versus* 218±59×10^3·mL^{-1} for non-ARDS; $p=0.07$); however, ARDS patients had a significantly higher percentage of neutrophils in BAL fluid (63±7% of total cell count in ARDS *versus* 22±7% in non-ARDS patients, $p<0.001$).

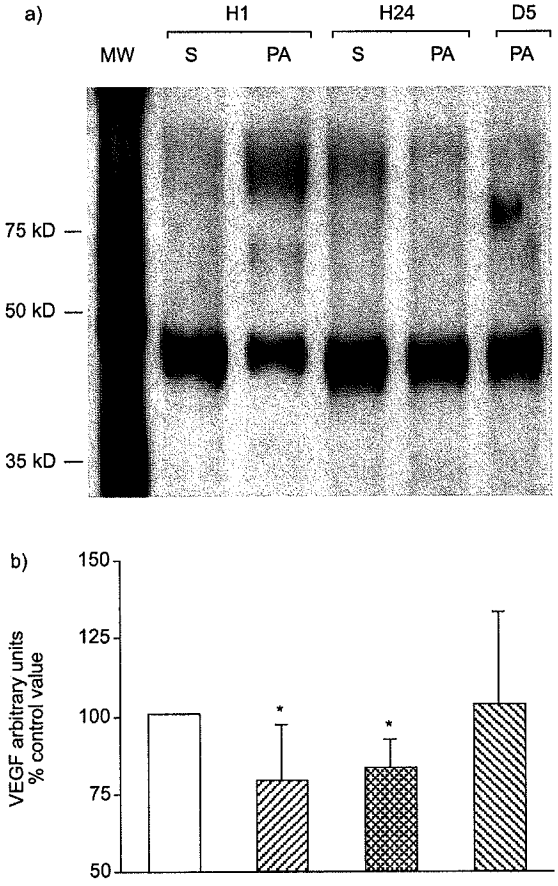

Fig. 4. – Effects of *Pseudomonas aeruginosa*-induced injury on vascular endothelial growth factor (VEGF) protein levels. a) Autoradiogram of signals for VEGF, showing three bands corresponding to the monomeric form and the dimeric form with or without glycosylation; and b) histogram showing a quantitative representation of VEGF protein levels obtained from laser densitometric analysis of four independent experiments. Results were expressed in arbitrary densitometric units. Cellular proteins were extracted from lung of sham (S, □) or *Pseudomonas aeruginosa*-instilled rats (PA) animals 4 h (H4, ▨), 24 h (H24, ▩) or 5 days (D5, ▩) after instillation. Proteins were analysed by immunoblotting and were fractionated on sodium dodecylsulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with a polyclonal VEGF antibody, as described in Materials and methods. MW: molecular weight markers. *: $p<0.05$ *versus* sham condition at the same time (H4 and H24).

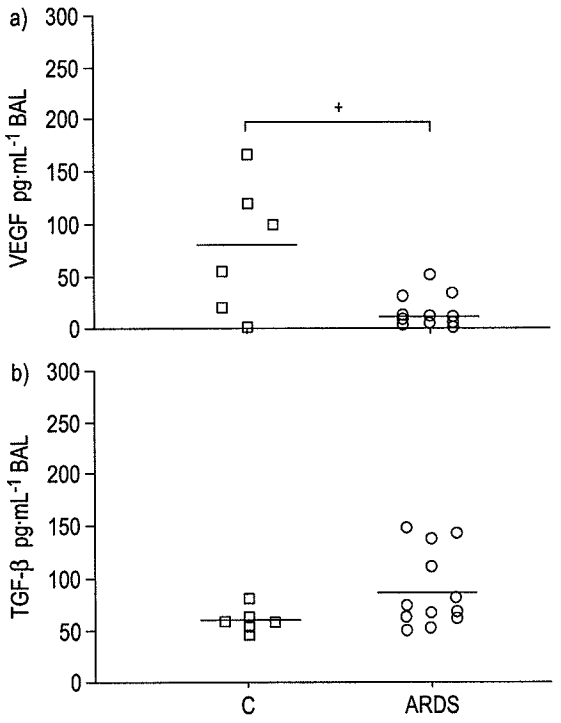

Fig. 5. – a) Vascular endothelial growth factor (VEGF) 165 and b) transforming growth factor-β1 (TGF-β1) levels in bronchoalveolar lavage (BAL) fluid of patients suffering from acute respiratory distress syndrome (ARDS) and of control patients (C). ○ : cytokine values of individual patients who had BAL sampling at the early phase of ARDS. □ : the individual values obtained in lavage fluids from control patients; —: the geometric means of each group. VEGF and TGF-β1 were measured by immunoassay as described in Materials and methods. +: $p=0.03$ for the comparison between ARDS and control patients.

Vascular endothelial growth factor and transforming growth factor-β1 levels in bronchoalveolar lavage from patients with and without acute respiratory distress syndrome

VEGF levels in BAL fluid were lower in the 13 ARDS patients than in the six non-ARDS patients (14.3 ± 11.1 pg·mL^{-1} *versus* 76.8 ± 51.1 pg·mL^{-1}, $p=0.03$) (fig. 5a). As expected, VEGF levels normalized for ELF were significantly decreased in ARDS patients (421 ± 245 pg·mL^{-1} *versus* $5,740\pm1,582$ pg·mL^{-1}, $p=0.001$). Similar results were observed when the mass of VEGF was calculated between the control and the ARDS groups (517 ± 245 ng·100 mL^{-1} *versus* $5,741\pm1,583$ ng·100 mL^{-1} of BAL, $p<0.001$). A trend was observed toward a correlation between VEGF concentration in ELF and the day of BAL procedure during ARDS course ($r_s=0.10$), but only three patients had a second BAL during their stay. Serum VEGF levels did not differ significantly between ARDS and non-ARDS patients (ARDS: 227 ± 152 pg·mL^{-1}, $n=9$ *versus* 649 ± 454 pg·mL^{-1}, $n=6$, NS).

In contrast, TGF-β1 levels in BAL fluids were slightly elevated in ARDS patients as compared to non-ARDS patients (85.6 ± 26.2 *versus* 59.2 ± 6.9, $p=0.07$) (fig. 5b); interestingly, TGF-β1 concentration was reduced when normalized for ELF (ARDS, $1,777\pm369$ pg·mL^{-1} *versus* non-ARDS, $7,667\pm3,440$ pg·mL^{-1}; $p=0.01$). No correlation was found between VEGF and TGF-β1. Neither VEGF nor TGF-β1 differed significantly between the direct injury and indirect injury ARDS subgroups.

Discussion

To investigate lung production of VEGF during acute lung injury (ALI), VEGF protein and mRNA

were measured in a rat model of severe bacteria-induced ALI previously developed in the laboratory [10]. This model has a 24-h mortality of 50%, and surviving rats exhibit severe hypoxaemia ($P_{a,O_2}/F_{I,O_2}$ ratio ~100 mmHg), marked protein leakage in BAL fluid, increased extravascular lung water, and diffuse alveolar damage [10, 11]. In the present study, VEGF protein and mRNA were decreased during the initial phase of ALI, at least from the fourth to the 24th hour, compared with control rats. The presented data from a severe rat ALI model are confirmed by the finding that VEGF was reduced in the BAL fluid at the initial phase of lung injury in patients with ARDS. Patients with ARDS were compared to those who were free of pulmonary oedema, but were treated with mechanical ventilation. A significant amount of VEGF protein was detected in BAL fluid from non-ARDS patients, without increased alveolar-capillary barrier permeability or lung injury. VEGF levels in ELF were higher than in serum, where platelets may be a major source [16], suggesting local production by the lung and a physiological role for VEGF in the normal lung. VEGF is an angiogenic and permeability increasing factor that is secreted by almost all the cell types found in airspaces or their lining during ARDS, namely alveolar epithelial cells, macrophages, and polymorphonuclear neutrophils. It was speculated that the VEGF present in BAL fluid may have been secreted mainly by alveolar type 2 cells since VEGF immunolocalization and mRNA expression reportedly occurs primarily in this cell type in the normal lung [17, 18], and it could act as a physiological survival factor for endothelial cells *via* a paracrine effect.

At least two mechanisms may be proposed to explain VEGF downregulation during ALI. Firstly, direct injury of alveolar epithelial cells may lessen the main source of VEGF in the lung by decreasing the number of cells producing VEGF. However, such a mechanism is unlikely since: 1) TUDER *et al.* [3] reported a decrease in VEGF mRNA after intraperitoneal injection of lipopolysaccharide (LPS) in rats, a model in which epithelial injury does not occur [3]; and 2) the present authors previously demonstrated that alveolar epithelial clearance was stimulated in the bacteria-induced ALI model, suggesting that alveolar type 2 cells are still functional in this model [10]. Secondly, the decrease in VEGF synthesis could constitute a lung response to different kinds of injuries, whether or not they induce epithelial damage. Along this line, acute hyperoxia-induced lung injury, whose charasteristic is endothelial rather than epithelial injury, is also associated with VEGF downregulation [19]. Monocrotalline administration in rats leading to pulmonary hypertension also decreases lung VEGF protein expression [20].

Downregulation of VEGF synthesis by alveolar epithelial cells could have occurred, due to the effect of inflammatory cytokines or cellular proliferation. Most inflammatory cytokines, which are known to be elevated in BAL fluid of ARDS patients [21, 22], stimulate VEGF expression in a variety of cultured cells [23–25] and, consequently, do not explain the decrease in VEGF in BAL fluid of the ARDS patients. Therefore, TGF-β was measured in BAL because this growth factor is known to be one of the most potent inducers of VEGF synthesis by epithelial cells [26]. Although the TGF-β levels were higher in BAL fluid in ARDS than in control patients, the TGF-β levels in ELF were lower in the ARDS patients. This suggests that the low level of VEGF in ARDS patients may be due to impaired TGF-β synthesis. However, a correlation was not found between the level of these two factors. This finding stresses the fact that a moderate variation of any marker has to be interpreted with caution when patients with very different volumes of ELF are compared, even if the method used to measure these volumes has limitations [27].

The finding of a downregulation of a vascular permeability factor, VEGF, was unexpected. The decrease in VEGF may protect lungs from alveolar flooding. In a recent study using an adenovirus-mediated gene transfer, KANER *et al.* [28] have shown that VEGF overexpression induces pulmonary oedema. Since VEGF is a permeability increasing factor, the decreased VEGF production demonstrated in the present study does not exclude its involvement in the early increase of endothelial permeability that may occur concomitantly with extravascular neutrophil migration at the onset of ARDS. Indeed, VEGF is stored in polymorphonuclear leukocyte granules [5, 6] and is probably secreted during neutrophil migration across the endothelium rather than within the airspaces. Moreover, this downregulation could participate in the well-known, but unexplained, decrease in capillary number and volume reported in the earliest histological description of ARDS [7]. It can also be speculated that decreased VEGF expression during the initial phase of ALI may be due to type 2 cell proliferation and may contribute to the initiation of alveolar re-epithelialization. The fibroproliferative response seen in ARDS has been shown to occur within a few days of the injury [29], and recent studies have found elevated levels of two growth factors for alveolar type 2 cells: hepatocyte growth factor and transforming growth factor-α in BAL fluid from patients with ARDS [30, 31]. These results point out the potential importance of epithelial factors in the early phase of ALI.

In conclusion, the vascular endothelial growth factor protein decrease in a rat model of severe acute lung injury was confirmed by findings from bronchoalveolar lavage fluids of patients with early acute respiratory distress syndrome. It is speculated that alveolar vascular endothelial growth factor downregulation may protect the lungs from alveolar flooding, participate in the decrease in capillary number, and contribute to the initiation of alveolar re-epithelialization.

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Simultaneous or Delayed Administration of Hepatocyte Growth Factor Equally Represses the Fibrotic Changes in Murine Lung Injury Induced by Bleomycin

A Morphologic Study

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Hepatocyte growth factor (HGF) is a humoral mediator of epithelial-mesenchymal interactions, acting on a variety of epithelial cells as mitogen, motogen, and morphogen. Exogenous HGF acts as a hepatotrophic factor and a renotrophic factor during experimental injury. To investigate whether HGF has a pulmotrophic function, human recombinant HGF was administered to C57BL/6 mice with severe lung injury by bleomycin (BLM). Low dose simultaneous and continuous administration of HGF (50 μg/mouse/7 d) with BLM (100 mg/mouse/7 d) repressed fibrotic morphological changes at 2 and 4 wk. Ashcroft score showed a significant difference in lung fibrosis with and without HGF at 4 wk (3.7 ± 0.4 versus 4.9 ± 0.3, $p < 0.05$). Furthermore, either simultaneous or delayed administration of high dose HGF (280 μg/mouse/14 d) equally repressed fibrotic changes by BLM when examined at 4 wk (Ashcroft score: 2.6 ± 0.4 and 2.4 ± 0.2 versus 4.1 ± 0.2, $p < 0.01$). Hydroxyproline content in the lungs was significantly lower in mice with either simultaneous or delayed administration of high dose HGF as compared to those administered BLM alone (121.8 ± 8.1% and 113.2 ± 6.2% versus 162.7 ± 4.6%, $p < 0.001$). These findings indicate that exogenous HGF acts as a pulmotrophic factor *in vivo* and prevents the progression of BLM-induced lung injury when administered in either a simultaneous or delayed fashion. HGF may be a potent candidate to prevent or treat lung fibrosis.

Yaekashiwa M, Nakayama S, Ohnuma K, Sakai T, Abe T, Satoh K, Matsumoto K, Nakamura T, Takahashi T, Nukiwa T. Simultaneous or delayed administration of hepatocyte growth factor equally represses the fibrotic changes in murine lung injury induced by bleomycin: a morphologic study. AM J RESPIR CRIT CARE MED 1997;156:1937–1944.

Hepatocyte growth factor (HGF) has been purified from the serum of partially hepatectomized rats (1), rat platelets (2), and human plasma (3). Notwithstanding the original concept of HGF as a potent mitogen for hepatocytes (1–4), it has been found to be a multifunctional growth factor, produced by mesenchymal cells such as endothelial cells (5, 6), fibroblasts (6, 7), and macrophages (6), and acts on a variety of epithelial cells and organs (8) as a mitogen (1, 4), motogen (9), and morphogen (10, 11). Thus, HGF is now recognized as a humoral mediator of epithelial-mesenchymal interactions (12). Recent findings indicate that HGF prevents apoptosis during embryogenesis and organogenesis in mice targeted with the HGF gene (13, 14). There is no species specificity among human, rat, and mouse with regard to biologic activities (15–18).

Administration of recombinant HGF following liver and kidney insults with hepatotoxins or renotoxins *in vivo* resulted in markedly elevated DNA synthesis in hepatocytes or renal tubular cells, suppression of hepatitis or renal damage, and stimulation of liver regeneration or reconstruction of the normal renal tissue structure (15–18). These findings suggest that HGF acts as a pleiotropic factor for various organs.

We reported that higher HGF levels were detected in the bronchoalveolar lavage fluid (BALF) of patients with idiopathic pulmonary fibrosis (19). Yanagita and coworkers reported that the HGF concentration in the sera of patients with lung diseases were higher than in those of normal controls (20, 21).

In vitro, HGF stimulates DNA synthesis in alveolar type II cells (22, 23). The receptor for HGF (c-*met* protooncogene product [24]) was expressed in alveolar type II cells but not in macrophages (22, 23), and the HGF-mRNA was detected in macrophages but not in type II cells (19, 22). In acute lung injury caused by hydrochloric acid (HCl), DNA synthesis in alveolar type II cells and increased HGF activity in the lung were observed *in vivo* (20).

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The information noted above suggests that HGF may act as a pulmotrophic factor. To determine whether exogenously administered HGF can protect against lung injury, human recombinant HGF was given to mice with severe lung injury induced by bleomycin (BLM), and the protective effect of HGF, administered simultaneously or after BLM, on this injury was analyzed.

METHODS

Animals and Materials

Female C57BL/6 mice (specific pathogen free), 8 to 10 wk of age, were purchased from Charles River Japan, Inc. (Yokohama, Japan), and were maintained at constant temperature (22° C), humidity (40%), and light cycle (8:00 A.M. to 8:00 P.M.) with food and water *ad libitum*. Human recombinant HGF (hrHGF) was provided by Dr. Nakamura (Osaka University School of Medicine, Osaka, Japan). BLM was supplied by Nihonkayaku Co. (Tokyo, Japan). Other reagents are of analytic grade.

Administration of hrHGF and BLM

Administration of HGF and BLM were performed by constant intraperitoneal (intraperitoneally [i.p.]) and subcutaneous (subcutaneously [s.c.]) infusion, respectively, through osmotic minipumps (Model 1007D and 2001, respectively; Alza Co., Palo Alto, CA) as specified below. In mice anesthetized with thiopental sodium (150 mg/kg i.p.), the pump with BLM was implanted subcutaneously on the back, slightly posterior to the scapulae, and the pump with HGF or vehicle was implanted intraperitoneally through a midline skin incision, about 0.5 cm long, in the lower abdomen posterior to the rib cage. After the mice were killed, the pumps were examined to determine if they had delivered the entire dosage of their contents in each mouse.

Determination of Tissue HGF Concentration

Three groups of three mice were used. Group A was treated with HGF (50 μg/mouse dissolved in 100 μl of saline) by constant i.p. infusion through a minipump (Model 1007D), and BLM (100 mg/kg dissolved in 200 μl of saline) by constant s.c. infusion from an osmotic minipump (Model 2001). Group B was treated using the same protocol as group A except that the amount of HGF per mouse was 333 μg instead of 50 μg. Group C was treated with BLM alone. Mice were killed 3 d after initial treatment. Tissue of lung, liver, and kidney were homogenized on ice in four volumes of buffer composed of 10 mM Tris-HCl (pH 7.5), 2 M NaCl, 0.01% Tween-80, 1 mM phenylmethyl-sulfonyl fluoride, and 1 mM ethylenediaminetetraacetic acid. After centrifugation at 105,000 × *g* at 4° C for 1 h, the supernatant was used for HGF quantification by an enzyme-linked immunosorbent assay (ELISA) system (Institute of Immunology, Tokyo, Japan) (18). A mouse monoclonal antibody against hrHGF was used according to the manufacturer's protocol. This mouse monoclonal antibody has little cross-reactivity against mouse HGF; hence, mouse HGF was not detected in this ELISA system.

Animal Treatment

Simultaneous administration of low dose HGF with BLM. To induce fibrotic changes in the lungs of mice, C57BL/6 mice received BLM by a continuous s.c. infusion from an osmotic minipump (Model 2001), from Day 0 to Day 7, as originally described by Harrison and coworkers (25). The minipump was loaded with BLM (100 mg/kg, dissolved in 200 μl of saline). Mice received HGF (50 μg/mouse, dissolved in 100 μl of saline) by a continuous i.p. infusion through a minipump (Model 1007D), from Day 0 to Day 7, simultaneously with BLM. Two and 4 wk after initial treatment, the mice were killed with thiopental sodium injection (450 mg/kg i.p.), thoraces were opened, lungs were removed, and protective effects of HGF for fibrotic changes induced by BLM were analyzed (Figure 1).

Simultaneous administration of high dose HGF with BLM. The mice were injured by BLM from Day 0 to Day 7 as described above. At the same time, the mice received a total dosage of 280 μg/mouse of HGF over 14 d; 140 μg by continuous i.p. infusion through a minipump for 7 d followed by bolus injections of 20 μg/mouse/d for 7 successive days. Mice were killed at 4 wk after initial treatment of BLM. The lungs were removed and analyzed.

Delayed administration of high dose HGF after BLM insults. The mice in the delayed HGF administration group first received BLM (100 mg/kg, from Day 0 to Day 7) as described above. Then on Day 14, the mice were treated with a total dosage of 280 μg/mouse of HGF over 14 days; 140 μg by continuous i.p. infusion through a minipump from Day 14 to Day 21 followed by bolus injections of 20 μg/mouse/d from Day 22 to 28. Mice were killed 4 wk after initial treatment of BLM. The lungs were removed and analyzed.

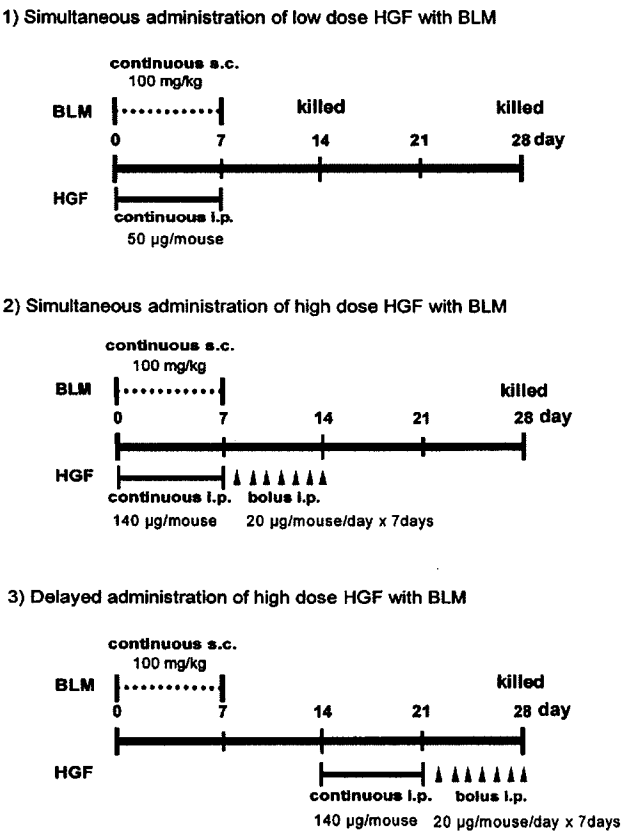

Figure 1. Experimental design of three different administration regimens of HGF and BLM. Mice in all groups received BLM by a continuous subcutaneous infusion from an osmotic minipump for 7 d, from Day 0 to 7. HGF was administered in three different ways as follows: (*1*) simultaneous administration of low dose HGF (50 μg/mouse, continuous i.p. administration for 7 d, from Day 0 to Day 7). Mice were killed 2 and 4 wk after initial treatment of BLM. (*2*) Simultaneous administration of high dose HGF (total dosage of 280 μg/mouse of HGF over 14 d; 140 μg by continuous i.p. infusion for 7 d, from Day 0 to Day 7, and followed by bolus i.p. injections of 20 μg daily for 7 successive days, from Day 8 to Day 14). Mice were killed 4 wk after initial treatment of BLM. (*3*) Delayed administration of high dose HGF (total dosage of 280 μg/mouse of HGF over 14 d; 140 μg by continuous i.p. infusion for 7 d, from Day 14 to Day 21, and followed by bolus i.p. injections 20 μg daily for 7 successive days, from Day 22 to Day 28). Mice were killed 4 wk after initial treatment of BLM.

Assessment of the Protective Effect of HGF on Lung Injury Induced by BLM

Histologic examination. The left lung was fixed by simple immersion in 10% buffered formaldehyde under constant negative pressure (10 cm water pressure) for 48 h in preparation for histologic examination.

TABLE 1

TISSUE CONCENTRATION OF EXOGENOUS HUMAN RECOMBINANT HGF IN MICE

HGF	n	Concentration in Organ Tissue (*ng/g*)		
		Lung	Liver	Kidney
HGF (50)*	3	0.63 ± 0.27	5.9 ± 2.4	1.6 ± 0.18
HGF (333)†	3	2.0 ± 0.04	47.2 ± 14.3	12.1 ± 0.71
Control‡	3	n.d.§	n.d.	n.d.

*, † Mice received BLM (100 mg/kg/7 d by continuous s.c. administration) and HGF (50 μg/7 d and 333 μg/7 d by continuous i.p. administration) through minipumps, and were killed on Day 3.

‡ Mice were treated with BLM alone as above through a minipump and killed on Day 3.

§ Not detected.

Inflation-fixation method was avoided because of technical problems to apply only to the left mouse lung. The fixed lung was sectioned sagittally, embedded in paraffin, and stained with elastica-Masson.

Quantitative evaluation of histologic findings. For the quantitative histologic analysis of fibrotic changes induced by BLM, a numerical fibrotic scale (Ashcroft scale [26]) was used. A numerical fibrotic score (Ashcroft score) was obtained as follows. The severity of the fibrotic changes in each lung section was assessed as a mean score of severity from observed microscopic fields. More than 25 fields within each lung section were observed at a magnification of ×100 in each successive field, and each field was assessed individually for the severity of fibrotic changes and allotted a score from 0 (normal) to 8 (total fibrosis) using a predetermined scale of severity (numerical fibrotic scale). After examination of the whole section, the mean of the scores from all fields was taken as the fibrotic score. In order to prevent observer's bias, all histologic specimens were randomly numbered and interpreted in a blinded fashion. Each specimen was scored independently by two or three observers, including a histopathologist; finally, the mean of their individual scores was considered as the fibrotic score.

Hydroxyproline analysis. Whole collagen content of the right lung was estimated by an assay of hydroxyproline (27). Briefly, after acid hydrolysis of the lung with 6 N HCl at 110° C for 16 h in a sealed glass tube (Iwaki, Tokyo, Japan), hydroxyproline content was determined by high performance liquid chromatography (28), and normalized by saline-treated control mice.

Statistical analysis. All values are shown as mean ± SEM. Statistical analysis of the results was performed with ANOVA and post hoc analysis with Newman-Kuels procedure (29). p Values lower than 0.05 were considered significant.

RESULTS

Tissue HGF Concentration

To assess the distribution of intraperitoneally administered exogenous hrHGF, tissue hrHGF concentrations in mice were determined by ELISA (Table 1). The tissue HGF concentra-

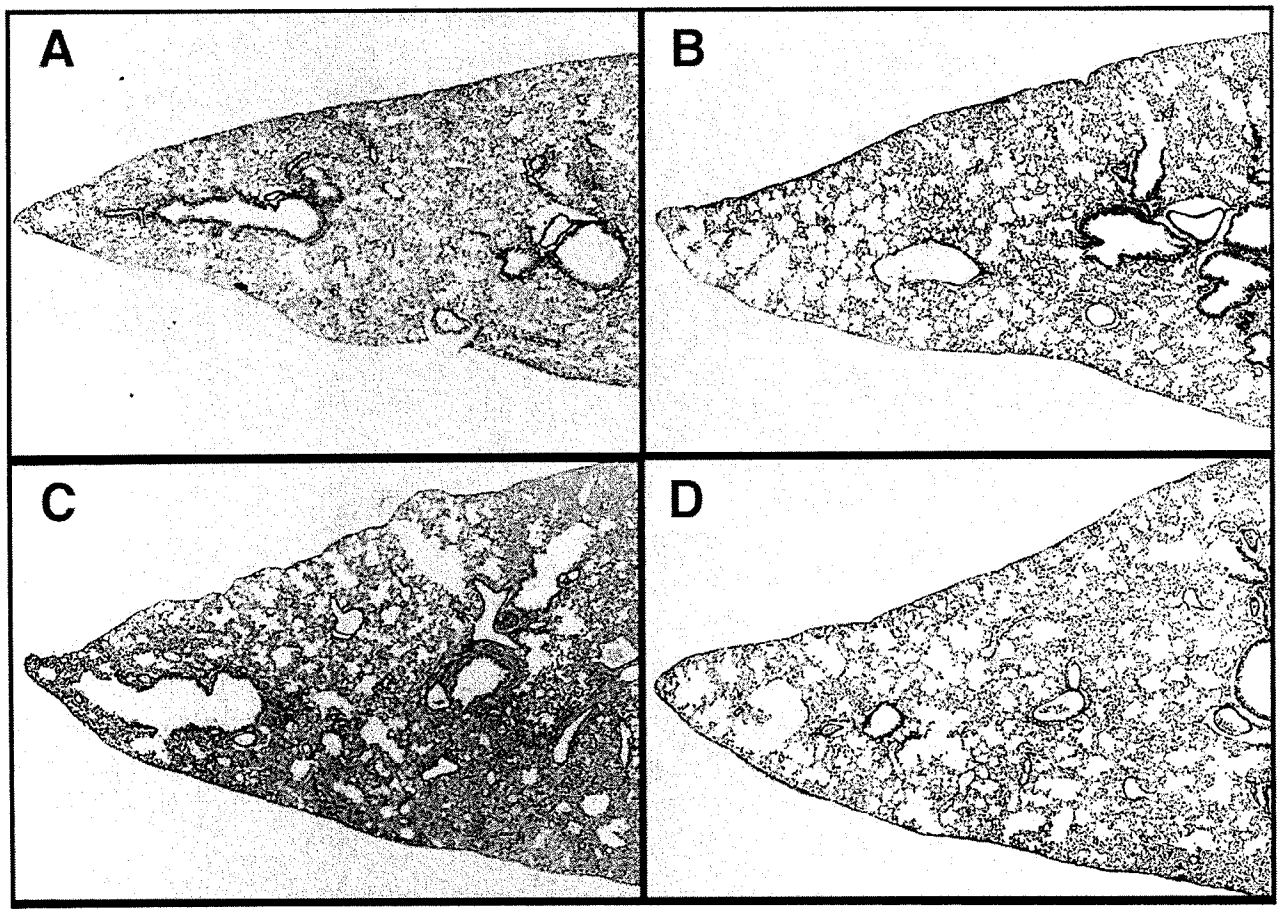

Figure 2. Microscopic findings of the murine lungs 2 and 4 wk after initial treatment of BLM and low dose HGF. (*A*) BLM (100 mg/kg, continuous s.c. administration from Days 0 to 7) alone at 2 wk; (*B*) BLM and low dose HGF (50 μg/mouse, continuous i.p. administration from Days 0 to 7) at 2 wk; (*C*) BLM alone at 4 wk; (*D*) BLM and low dose HGF at 4 wk. Lungs were stained with elastica-Masson. Original magnification: ×10.

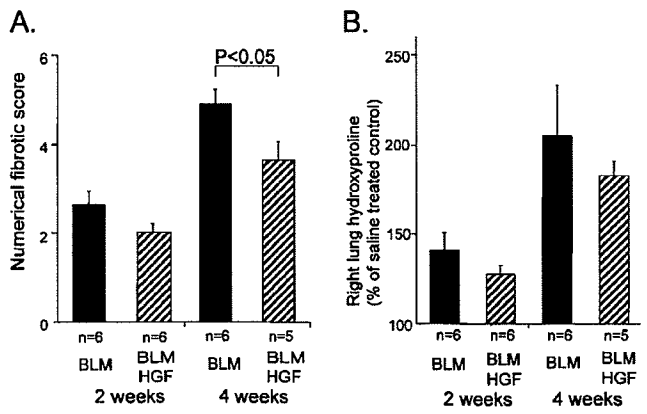

Figure 3. Evaluation of fibrotic changes in the lung by numerical fibrotic score and measurement of hydroxyproline content in the lung treated with BLM and low dose HGF. (*A*) Evaluation of fibrotic changes by a numerical fibrotic scale (Ashcroft scale) in the murine lung treated with BLM and low dose HGF. Fibrotic score (Ashcroft score [26]) was obtained with a continuous numerical scale for determining the degree of fibrotic changes in the lung. Grade was scored on a scale from 0 to 8, with use of the average of microscopic field scores. The average score of the group treated with BLM and low dose HGF (*hatched bars*) was lower than that with BLM alone (*closed bars*) both at 2 and 4 wk after initial treatment of BLM. There was a significant difference between these two groups at 4 wk when examined by ANOVA and post hoc analysis with Newman-Kuels procedure ($p < 0.05$). (*B*) Measurement of hydroxyproline content in the lung treated with BLM and low dose HGF. BLM (100 mg/kg, continuous s.c. administration) and vehicle (saline) (*closed bars*) or BLM and low dose HGF (50 μg/mouse, continuous i.p. administration) (*hatched bars*) were administered for 7 d. Lungs were removed 2 and 4 wk after initial treatment of BLM. Hydroxyproline content in the right lung was measured and normalized to those obtained from mice treated neither by BLM nor HGF (saline treated control mice).

tion after 3 d of continuous i.p. administration showed that only a small fraction was distributed. The lung contained less hrHGF per gram of tissue than liver and kidney. In addition, less proportional increase of hrHGF in the lung between group A (hrHGF 50 μg/mouse/7 d) and group B (hrHGF 333 μg/mouse/7 d) was observed in contrast to those in the liver or kidney.

Protective Effect of hrHGF Administered Simultaneously with BLM

Morphologic findings. Murine lung injury was induced by BLM released from an osmotic minipump subcutaneously implanted in the mouse. The dose of hrHGF used in this study (50 μg/mouse/7 d i.p.) was comparable to those in the experiments in which the hepato- or renotrophic effects of HGF was evaluated (5 to 10 μg/mouse/d i.v.) (15–18).

Histologic findings in control mice treated with BLM alone showed that fibrotic changes were progressive at 2 and 4 wk after treatment. Examination 2 wk after initial treatment revealed focal fibrotic lesions primarily in subpleural and occasionally in perivascular areas with consolidation of lung parenchyma, loss of alveolar architecture, and increased cellularity with alveolar macrophages (Figure 2A). At 4 wk, fibrotic changes became more severe, expanding to the central areas of the lobes, involving the perivascular and peribronchiolar regions, and showing much more confluence and uniformity of appearance in subpleural areas (Figure 2C).

In contrast to the findings in mice treated with BLM alone, histologic findings at 2 wk with simultaneous administration of HGF (50 μg/mouse/7 d) revealed that fibrotic lesions were less focal in the subpleural areas, and fibrotic changes were milder in degree than in mice treated with BLM alone (Figure 2B). After 4 wk, focal fibrotic changes, which did not expand to the central areas of the lobe, were observed in subpleural areas and lung tissue outside of the subpleural areas appeared normal except for increased cellularity with alveolar macrophages (Figure 2D).

Quantitative evaluation of histologic change. The overall grades of the fibrotic changes of the lungs were obtained by the numerical score (Ashcroft score) 2 and 4 wk after treatment. The scores in the mice treated with BLM with simultaneous HGF and with BLM alone were 2.0 ± 0.2 and 2.6 ± 0.3 at 2 wk, and 3.7 ± 0.4 and 4.9 ± 0.3 at 4 wk, respectively (Figure 3A). All mice survived the experiment ($n = 6$ in each group). However, one mouse, treated with HGF and killed on day 28, received an insufficient dosage of HGF because of technical problems with the intraperitoneal minipump, and was excluded from this study. A significant difference in the scores between two groups was demonstrated at 4 wk by ANOVA and post hoc analysis with Newman-Kuels procedure ($p < 0.05$).

Assessment of fibrosis by hydroxyproline content. Collagen content was assessed by measuring hydroxyproline in the right lung 2 and 4 wk after initial treatment. The hydroxyproline content was lower in lungs treated with BLM and simultaneous HGF (Figure 3B). Control C57BL/6 mice which were treated with saline showed minor increases in pulmonary hydroxyproline content (data not shown). In this context, hydroxyproline content in the right lungs, normalized by saline-treated control mice, 2 and 4 wk after treatment was 127.5 ± 4.9% and 182.8 ± 8.3% in mice treated with BLM and HGF, and 140.9 ± 9.9% and 205.1 ± 28.0% in mice with BLM alone, respectively. This reduction of the hydroxyproline content was not statistically significant.

Comparison of Simultaneous and Delayed Administration with High Dose HGF on Lung Injury Induced by BLM

Morphologic changes. Simultaneous administration of BLM and HGF (50 μg/mouse/7 d) partly suppressed the fibrotic change. A higher dose of HGF (total, 280 μg/mouse/14 d) given either simultaneously with BLM or 2 wk later, showed at 4 wk that the suppressive effect was obvious irrespective of simultaneous or delayed administration of HGF (Figure 4). Fibrotic changes in the lungs in both groups treated with higher dose HGF were limited to the subpleural areas and some parts of the perivascular or peribronchiolar areas. Other areas showed almost normal alveolar architecture. There were no specific histologic differences noted between these two groups.

Quantitative evaluation of histologic changes. Quantitative evaluation by a numerical fibrotic score (Ashcroft score) confirmed the suppressive effect of HGF on fibrotic changes when administered in either a simultaneous or delayed fashion. No mice died during the experiment. In this context, significant differences in the scores were observed when comparisons were made (*1*) between the group treated with BLM alone ($n = 5$) and that treated with BLM and simultaneous HGF ($n = 5$) (4.1 ± 0.2 versus 2.6 ± 0.4, $p < 0.01$), and (*2*) between the group treated with BLM alone and that treated with BLM and delayed HGF ($n = 5$) (4.1 ± 0.2 versus 2.4 ± 0.2, $p < 0.01$) (Figure 5A). The fibrotic scores of mice with higher dose HGF was significantly smaller than that of mice with low dose HGF ($p < 0.05$).

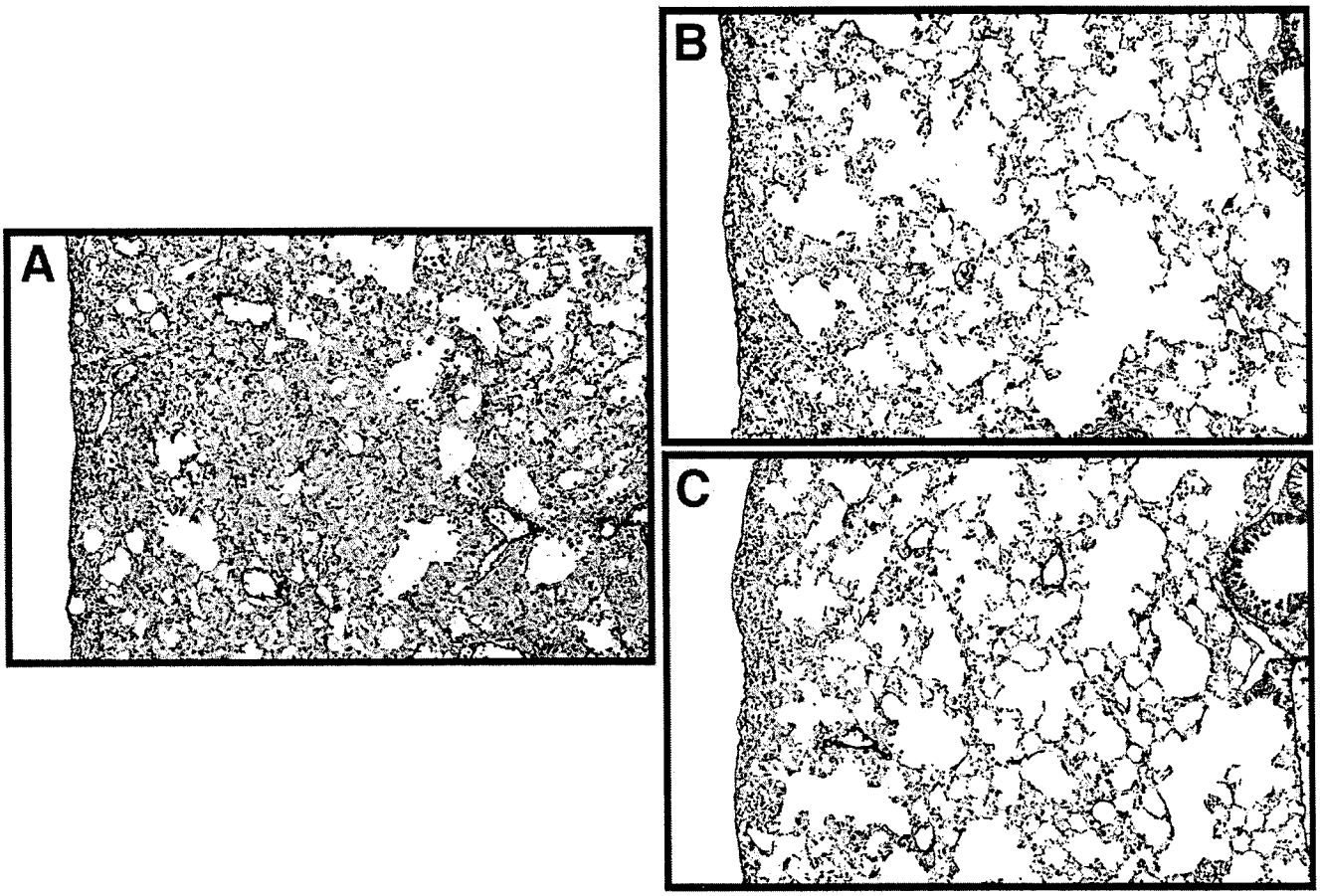

Figure 4. Microscopic findings of the murine lung injured by BLM and treated with simultaneous or delayed administration of high dose HGF. (*A*) With BLM alone; (*B*) with BLM (100 mg/kg, continuous s.c. administration from Days 0 to 7) and simultaneous high dose HGF (total dosage of 280 μg/mouse of HGF over 14 d; 140 μg by continuous i.p. infusion for 7 d, from Day 0 to Day 7, and followed by bolus i.p. injections of 20 μg daily for 7 successive days, from Day 8 to Day 14); (*C*) with BLM and delayed high dose HGF (total dosage of 280 μg/mouse of HGF over 14 d; 140 μg by continuous i.p. infusion for 7 d, from Day 14 to Day 21, and followed by bolus i.p. injections 20 μg daily for 7 successive days, from Day 22 to Day 28). The lungs were removed at 4 wk after initial treatment of BLM, and stained with elastica-Masson. Original magnification: ×40.

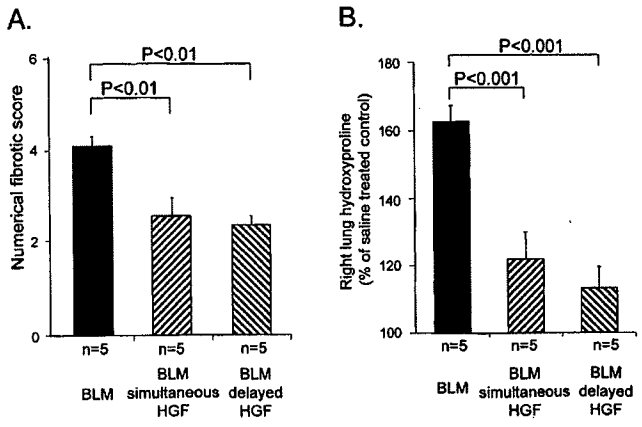

Figure 5. Evaluation of fibrotic changes in the lung by numerical fibrotic score and measurement of hydroxyproline content in the lung treated with BLM and simultaneous or delayed administration of high dose HGF. (*A*) Evaluation of fibrotic changes by numerical fibrotic scale (Ashcroft scale). Both of averages of numerical fibrotic scores of the group treated with BLM and simultaneous high dose HGF (*hatched bars*), and those treated with BLM and delayed high dose HGF (*hatched bars*) were significantly lower than those with BLM alone (*closed bars*) 4 wk after initial treatment of BLM. (*B*) Measurement of hydroxyproline content in the lung treated with BLM and high dose HGF. Hydroxyproline content in right lung of both groups treated with BLM and simultaneous (*hatched bars*) or delayed (*hatched bars*) administration of high dose HGF was significantly lower than those with BLM alone (*closed bars*) 4 wk after initial treatment of BLM. Hydroxyproline content in the right lung of mice was normalized to those in saline-treated controls. Statistical analysis of the results was performed with ANOVA and post hoc analysis with Newman-Kuels procedure.

Assessment of fibrosis by hydroxyproline content. Mice treated with high dose HGF and BLM were used for assessment of collagen content by measuring hydroxyproline in the right lung 4 wk after initial treatment. The hydroxyproline content in the lungs was significantly lower in groups treated with BLM and simultaneous or delayed administration of high dose HGF in comparison to the group treated with BLM alone ($p < 0.001$, $p < 0.001$) (Figure 5B). When normalized with saline-

treated control mice, hydroxyproline content in the lungs were 121.8 ± 8.1%, and 113.2 ± 6.2%, and 162.7 ± 4.6% in mice with simultaneous and delayed administration of high dose HGF, and BLM alone, respectively.

DISCUSSION

Mode of HGF Action in Repair Process of Damaged Respiratory Epithelial Structures

Organotoxins induce parenchymal cell death, and cause subsequent pathologic remodeling of normal structure, resulting in functional insufficiency of organs. HGF, as a multifunctional and essential cytokine transducing a superb signal for normal morphogenesis and regeneration, functions as an antiorganotoxic agent in several organs (12, 15–18, 30, 31). In the present study, we hypothesized that exogenous HGF may act as a pulmotrophic factor that prevents respiratory epithelial cell death as well as promoting ordered regeneration of the peripheral respiratory tract. In mice with simultaneous low dose HGF during BLM administration, there was a significant difference in suppressed fibrosis by morphology at 4 wk in comparison with BLM-treated mice, although collagen deposition assayed by hydroxyproline in mouse lung treated with HGF and BLM contained a reduced amount, but not significant. With high dose HGF, both simultaneous and delayed, HGF not only effectively suppressed fibrotic changes, but actually ameliorated them, even when administered 2 wk after the start of BLM treatment. Moreover, with high HGF dose, significant repression of fibrotic changes was confirmed by assessment of hydroxyproline. To date, very few reports have described the effective suppression of the fibrotic process when agents are administered after the onset of damage. Only prior and/or simultaneous administration of steroid hormones (32) or antibodies to cytokines such as anti-TNF-α antibody are capable of preventing damage (33, 34). Significance of this consequence in the clinical setting is obvious, because the treatment should be effective despite the fact that the clinical manifestation of lung injury or chronic inflammation usually becomes apparent after the latent period following insults.

Route of HGF Administration and the Concentration of HGF in the Tissue

In our experimental design, both BLM and HGF were administered by continuous infusion through osmotic minipumps. Despite the fact that excessive amounts of HGF (50 μg/mouse/7 d for low dose, and 280 μg/mouse/14 d for high dose) were used, only a small fraction of HGF may have reached the injured organs, because HGF binds to heparin in the peritoneum as well as to connective tissues along the route of diffusion. *In vitro*, HGF stimulates DNA synthesis in alveolar epithelial cells in a concentration-dependent manner, reaching a maximum at an HGF concentration of 5 to 10 ng/ml (35). In our study, the tissue concentration of HGF in the lung at Day 3 was 0.63 ± 0.27 ng/g tissue when low dose HGF (50 μg/7 d) was used, and 2.0 ± 0.04 ng/g tissue when higher dose HGF (333 μg/7 d) was used. Thus, we assumed that these HGF concentrations were capable of stimulating alveolar epithelial DNA synthesis.

A continuous osmotic infusion pump was chosen because of the difficulty with repeated intravenous administrations of HGF. Regarding the drug delivery system, however, the continuous infusion as well as the strong binding of HGF to heparin (1, 2) in the connective tissue allows for constant chronic dosing of HGF in mice. Because high local concentrations of HGF have not caused any serious adverse effect to date, these results provide a potential option for future treatment, perhaps even direct expression of HGF cDNA through gene therapy.

Lung Injury, Fibrosis, and Therapeutic Concepts for Repair

With regard to the mechanisms of lung damage and disordered remodeling (36, 37), therapeutic strategies can be conceptualized into three categories: (*1*) to counteract active inflammatory reactions caused by pro-inflammatory agents, (*2*) to avoid apoptotic loss of epithelial cells and stimulate ordered tissue repair, and (*3*) to eliminate chronic stimuli for fibrosis.

Current therapeutic approaches are mostly confined to the early inflammatory phase. Steroid hormones are effective in suppressing the activity of immune effector cells (38), anti-oxidant agents such as glutathione (GSH) are effective against oxidative stimuli (39), and antiproteases are active against proteolytic damage (40, 41). However, in addition to suppression of the pro-inflammatory and pro-injurious agents, strong therapeutic support for the repair of epithelial cells is essential. Potent growth factors for alveolar type II epithelial cells have been characterized, such as epidermal growth factor (EGF), transforming growth factor-α, (TGF-α), keratinocyte growth factor (KGF), acidic and basic fibroblast growth factor (aFGF, bFGF), and HGF (22, 23, 35, 42–44). Panos and colleagues have demonstrated that HGF and KGF induce DNA synthesis in alveolar type II cells, and HGF stimulates DNA synthesis more strongly than KGF (35). Similar results were reported by Shiratori and coworkers, i.e., HGF is more potent in stimulating DNA synthesis than aFGF, TGF-α, or EGF (23). In our study, simultaneous administration of HGF protected against lung injury induced by BLM, suggesting that HGF stimulated DNA synthesis in type II epithelial cells and promoted the turnover of damaged epithelial cells. This in turn would diminish inflammatory changes and suppress fibrotic changes in the lung. Recently, the possibility that HGF may be involved in the prevention of apoptosis was reported (13, 14). Matsumoto and colleagues have described HGF as a potent survival factor for pheochromocytoma PC12 cells in culture (45). Thus, HGF may have protective activity in damaged respiratory epithelial cells through an anti-apoptotic mechanism.

TGF-β1 is a very potent inducer of tissue fibrosis due to its chemotactic and stimulatory activity for matrix synthesizing cells (46–48). After administration of BLM, TGF-β1 mRNA and protein in lung tissue were elevated and peaked at 7 to 10 d (47). Subsequently, collagen and other extracellular matrix proteins peaked at 10 to 14 d (47, 49, 50). The timing of delayed administration of HGF in this study coincided with the time course of TGF-β1 involvement. HGF suppressed the fibrotic changes in lung even at this stage. Although HGF expression is dramatically inhibited by TGF-β1 (51, 52), exogenous HGF may affect the function of TGF-β1 directly or indirectly and may mitigate the tendency toward lung fibrosis (53). In this context, as a therapeutic approach, it is of interest to examine the synergistic effect of HGF and anti-TGF-β1 agents (48, 54).

In summary, our results suggest a substantial therapeutic benefit of HGF in both early and delayed BLM lung injury. Further long-term studies will be necessary to confirm the clinical efficacy of this therapy.

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Hepatocyte growth factor and neutrophil elastase in idiopathic pulmonary fibrosis.

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It has been hypothesized that hepatocyte growth factor (HGF) may play an important role in regulating the growth of lung epithelium and in the regeneration of the lung as a paracrine or endocrine factor in idiopathic pulmonary fibrosis (IPF). Based on this background, serum HGF was measured in 31 IPF patients (21 male/10 female, median age 60 years). Fifteen age-matched normal non-smokers served as the control. Hepatocyte growth factor was measured by enzyme-linked immunosorbent assay with monoclonal and polyclonal antibodies against human HGF (Otsuka Assay Laboratories, Tokushima, Japan). Elastase: alpha 1-proteinase complex was also measured by enzyme-linked immunosorbent assay. No patients had significant liver or renal dysfunction. As a result, mean (standard error) serum HGF concentration of the patients with IPF was 0.384 (0.022) ng ml-1, which was significantly high compared to normal non-smokers [0.213 (0.012) ng ml-1, $P < 0.001$, 95% confidence interval was between 0.104 and 0.238]. Serum HGF values correlated strongly with the plasma elastase: alpha 1 proteinase inhibitor complex ($R = 0.679$, $P < 0.001$). Immunohistochemical staining of lung tissue with anti-human neutrophil elastase showed scattered immunopositive cells mainly in interstitium. Immunohistochemical staining with mouse anti-human HGF antibody showed that HGF was distributed to the lung epithelial cells in IPF lung specimens obtained by open lung biopsy. These results suggest that HGF may play an important role in the pathogenesis of IPF.

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Measurement of hepatocyte growth factor in serum and bronchoalveolar lavage fluid in patients with pulmonary fibrosis.

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The present study evaluated the clinical significance of hepatocyte growth factor (HGF) in patients with pulmonary fibrosis. Twenty-one patients with a diagnosis of pulmonary fibrosis [14 with idiopathic pulmonary fibrosis (IPF) and seven with pulmonary fibrosis associated with a collagen vascular disorder (PF-CVD]) and 21 normal subjects as control were studied. HGF levels in sera of patients with pulmonary fibrosis (0.34 +/- 0.02 ng ml-1) were elevated significantly as compared with normal subjects (0.21 +/- 0.01 ng ml-1) ($P < 0.0001$). HGF/albumin levels in broncho-alveolar lavage fluid (BALF) of patients with pulmonary fibrosis (72 +/- 17 ng g-1 albumin) were also significantly elevated as compared with normal subjects (under the detection limit) ($P < 0.01$). HGF levels in sera correlated significantly with elastase levels in sera and C-reactive protein, and correlated negatively with PaO2. HGF levels in sera were significantly higher in smokers with pulmonary fibrosis (0.42 +/- 0.03 ng ml-1) as compared with non-smokers with pulmonary fibrosis (0.29 +/- 0.03 ng ml-1) ($P < 0.005$). HGF/albumin levels in BALF correlated significantly with elastase/albumin levels in BALF, lactate dehydrogenase/albumin in BALF, Immunoglobulin A/albumin in BALF, total cell count/albumin in BALF, total number of alveolar macrophage/albumin in BALF, total number of neutrophil/albumin in BALF, CEA/albumin in BALF, CA19-9/albumin in BALF, and SCC/albumin in BALF. These results suggest that following lung injury, HGF may be a mediator involved in the repair which leads to pulmonary fibrosis.

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Links

Hepatocyte growth factor in bronchoalveolar lavage fluids and cells in patients with inflammatory chest diseases of the lower respiratory tract: detection by RIA and in situ hybridization.

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Pulmonary fibrosis is a chronic inflammatory disorder characterized by diffuse fibrous remodeling of alveolar spaces. Although much interest is focused on mechanisms of the inflammatory process in pulmonary fibrosis, little is known about the repair and regenerative process. Hepatocyte growth factor (HGF), originally discovered as a mitogen for hepatocyte regeneration, is now recognized as a multifunctional mesenchymal factor for epithelial regeneration, including the regeneration of alveolar type II epithelial cells. Involvement of HGF and its receptor (c-met) is evident in animal models of acute lung injury produced by hydrochloride inhalation. We studied the role of HGF in patients with idiopathic pulmonary fibrosis (IPF) (25 cases), lung fibrosis associated with rheumatoid arthritis (22 cases), and sarcoidosis (39 cases). Immunohistochemical evaluation demonstrated that hyperplastic alveolar type II epithelial cells, as well as alveolar macrophages, were strongly stained with anti-HGF antibody in tissues of patients with IPF. The *concentration of HGF in bronchoalveolar lavage fluid* (BALF) was significantly higher than in normal controls (0.23 +/- 0.09 pg/microg) in patients with IPF (0.77 +/- 0.88 pg of HGF/microg of albumin, $P < 0.001$), lung fibrosis associated with rheumatoid arthritis (0.50 +/- 0.64 pg/microg, $P < 0.01$), and sarcoidosis (0.41 +/- 0.61 pg/microg, $P < 0.05$). In situ hybridization revealed mRNA for HGF in alveolar macrophages (especially small monocytelike macrophages). These results indicate that the increase in HGF concentration in patients' peripheral air spaces is due to augmented HGF production by alveolar epithelial cells and alveolar macrophages. HGF, through a paracrine mechanism, may play an important role in the

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Brief Communication

Decreased Level of Vascular Endothelial Growth Factor in Bronchoalveolar Lavage Fluid of Normal Smokers and Patients with Pulmonary Fibrosis

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Vascular endothelial growth factor (VEGF) plays multifunctional roles in both the development of vasculature and the maintenance of vascular function. A decrease in VEGF reduces angiogenesis and induces apoptosis of vascular endothelial cells. Inhibition of the VEGF receptor causes endothelial cell apoptosis and emphysema. We postulated that VEGF concentrations might be reduced in patients with chronic lung diseases. The level of VEGF was evaluated by enzyme-liked immunosorbant assay in bronchoalveolar lavage fluid (BALF) from normal smokers, nonsmoking volunteers, idiopathic pulmonary fibrosis, pulmonary fibrosis associated with a connective tissue disease, and sarcoidosis. The isoforms of VEGF in BALF were determined by high-performance liquid chromatography. VEGF in nonsmoking volunteers was detectable at a high concentration. In contrast, VEGF in most of the normal smokers was below the detectable limit. The VEGF found in nonsmoking volunteers BALF was VEGF165. VEGF was significantly decreased in idiopathic pulmonary fibrosis, pulmonary fibrosis associated with a connective tissue disease, and sarcoidosis compared with nonsmoking volunteers. The smoking patients showed a further decrease in VEGF. These data suggest that the decrease in VEGF in smokers and patients with chronic lung diseases may reduce angiogenesis and induce apoptosis of vascular endothelial cells.

Keywords: vascular endothelial growth factor; bronchoalveolar lavage; smoking

Vascular endothelial growth factor (VEGF) is a dimorphic glycoprotein with a molecular weight of 34,000 to 42,000, consisting of two disulfide-linked peptide chains having identical N termini (1, 2). VEGF possesses potent vascular permeability-enhancing activity. On a molar basis, it is 50,000 times more potent than histamine (1). VEGF was first identified in tumor cell-conditioned media (3) and has been purified to homogeneity from guinea pig and human sources (1, 4). VEGF acts directly on cultured vascular endothelium to induce a rapid increase in free cytosole calcium, apparently by activating a phosphoinositide-specific phospholipase C (5). It stimulates the release of von Willebrand factor from endothelial cells (5) and induces the expression of endothelial cell tissue factor activity (6). Concurrently with these studies, a protein selectively mitogenic for vascular endothelial cells was purified from cell culture, and this protein turned out to be the same substance that induced vascular permeability-enhancing activity (7, 8). VEGF is chemotactic for endothelial cells (9) and enhances collagenase (10) and urokinase receptor expression in endothelial cells (11). The existence of multiple activities embodied in the same protein suggests that VEGF may play multifunctional roles in both the development of vasculature and the maintenance of vascular structure and function.

The gene for human VEGF is organized into eight exons. As a result of alternative splicing, at least four transcripts have been detected, encoding mature monomeric VEGF containing 121, 165, 189, and 206 amino acid residues (VEGF121, VEGF165, VEGF189, and VEGF206), each preceded by a 26-amino acid signal peptide (12). VEGF121 and VEGF165 are diffusible proteins that are secreted into medium. VEGF189 and VEGF206 have high affinity for heparin and are mostly bound to heparin-containing proteoglycans in the extracellular matrix (13). Transfection studies demonstrate that VEGF121 and VEGF165 are secreted and have both mitogenic and peameability-inducing activities. In contrast, VEGF189 and VEGF206 remain primarily cell associated and lack the mitogenic activity of the smaller form (14, 15), suggesting that VEGF165 and VEGF121 are pivotal forms of VEGF. Among several peptide growth factors implicated in angiogenesis, including transforming growth factor-β, platelet-derived growth factor, and VEGF, only VEGF is specifically mitogenic for endothelial cells, having no mitogenic activity for other cell types (16). VEGF mRNA has been found in embryonic tissues, including lung, kidney, and placenta, during organ angiogenesis (14, 17). Although VEGF transcripts are located in epithelial cells, smooth muscle cells, and macrophages in various tissues, in normal adult lung, expression has been mainly in alveolar epithelial cells (18).

The mechanisms that maintain lung alveolar capillary structure and function are not completely known. Recently, Kasahara and coworkers reported that inhibition of VEGF receptors causes endothelial cell apoptosis and emphysema, suggesting that VEGF receptor signaling is required for maintenance of the alveolar structure and that alveolar septal cell apoptosis contributes to the pathogenesis of emphysema (19). If VEGF is necessary for the maintenance of healthy pulmonary capillary endothelium and blocking its receptor causes emphysema, then a reduced level of VEGF in the epithelial lining fluid from smoking may be a mechanism whereby cigarette smoking causes emphysema by inducing endothelial apoptosis and alveolar wall destruction. Thus, in this study, we evaluated whether smoking and other chronic lung diseases are associated with a reduction in the concentration of VEGF in bronchoalveolar lavage fluid (BALF).

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METHODS

Normal Control Subjects

Analysis of VEGF and cellular constituents of BALF was performed in 27 healthy volunteers and in patients with idiopathic pulmonary fibrosis (IPF), pulmonary fibrosis associated with a connective tissue disease (PF-CTD), and sarcoidosis (Sar) (Table 1). Sixteen were never-smokers, and 11 were current smokers. Smoking history varied from 5.5 to 13.4 years (8.9 ± 3.8 years, average). They smoked 1.2 ± 0.3 packs per day. These subjects had no respiratory symptoms, normal chest radiographs, and normal spirometric test results, lung volume, and diffusing capacity for carbon monoxide. None of the subjects had evidence of hypersensitivity pneumonia, left ventricular dysfunction, cardiac valvular diseases, or significant occupational exposures. Informed consent was obtained from each patient and healthy volunteer, and the protocol was approved by the Institutional Human Subjects Review Committee.

The patients with IPF, PF-CTD, and Sar were admitted or referred to the Chest Disease Research Institute of Kyoto University. Consecutive patients participated in this study after diagnosis was determined, regardless of the severity of diseases and acute or chronic (stable) phase.

Patients with IPF

Fourteen patients with IPF were evaluated (Table 1). IPF was diagnosed on the basis of a comparable history, physical examination, chest radiograph, and pulmonary physiologic evaluation. A confirmatory open-lung biopsy was performed in all 14 subjects. Thirteen out of 14 patients had usual interstitial pneumonia (UIP), and one of the patients had nonspecific interstitial pneumonia. Two of the patients with IPF were receiving treatment at the time of lavage (prednisolone alone). Eleven patients were either never-smokers or ex-smokers (quit at least 6 months before), and there were three current smokers.

Patients with PF-CTD

Twenty-one patients with PF-CTD were examined. Seven patients had progressive systemic sclerosis (seven, UIP). Six had dermatomyositis/polymyositis (four, UIP; two, nonspecific interstitial pneumonia). One had mixed connective tissue disease (one, UIP). Two had rheumatoid arthritis (one, UIP; one, bronchiolitis obliterance organizing pneumonia), and five had Sjogren's syndrome (four, UIP; one, bronchiolitis obliterance organizing pneumonia). Ten patients with PF-CTD were receiving treatment at the time of lavage (prednisolone alone). Sixteen patients were never-smokers or were ex-smokers (quit at least 6 months before), and five patients were current smokers.

Patients with Sar

Fourteen patients with Sar participated in this study. These patients were diagnosed on the basis of a comparable history, physical examination, chest radiograph, typical BALF lymphocyte surface markers, and pulmonary physiologic evaluation. A confirmatory open-lung biopsy was performed in 14 subjects. Two patients were treated with oral steroids. Nine patients were never-smokers or ex-smokers (quit at least 6 months before), and five patients were current smokers.

TABLE 1. CHARACTERISTICS OF THE STUDY SUBJECTS

Study Groups	n	Age (*yr*)	Male/Female	Serum LDH Concentration (*IU/L*)
Nonsmoking volunteers	16	44.9 ± 6.8	15/1	ND
Normal smokers	11	48.1 ± 5.8	8/3	ND
IPF	14 (3)	66.0 ± 8.4	14/0	471.2 ± 29.9
PF-CTD	21 (5)	52.5 ± 5.0	8/13	464.3 ± 35.1
Sar	14 (5)	40.3 ± 10.1	10/4	311.1 ± 18.9

Definition of abbreviations: IPF = idiopathic pulmonary fibrosis; LDH = lactate dehydrogenase; PF-CTD = pulmonary fibrosis with a connective tissue disease; Sar = sarcoidosis.

Age, mean ± SEM. The number of smoking patients was shown in the parentheses. Normal range of LDH, 230–460 IU/L.

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed in an outpatient setting using previously reported methods (20). Differential counts on 400 cells were performed on Wright-Giemsa–stained preparations made from the pooled lavage fluids before centrifugation. The pooled lavage fluid was centrifuged at 400 × *g* (2,000 rpm) for 10 minutes to separate the cells from the supernatant. The supernatant was stored at −80°C until assayed.

Measurement of VEGF Protein in BALF

The concentrations of VEGF and albumin in the unconcentrated BALF were assayed by an enzyme-linked immunosorbant assay kit (Amersham, Little Chalfont, UK) and the bromocresol green method (Seiken, Tokyo, Japan), respectively. The minimum concentration of VEGF detected by this method was 15.6 pg/ml. Then we calculated VEGF concentration divided by albumin concentration (pg/mg) in BALF.

Determination of the Isoform of VEGF in BALF by High-Performance Liquid Chromatography

To determine the isoforms of VEGF in BALF, we used high-performance liquid chromatography (Waters 2690 separations Module; Waters Corp., Milford, MA) by using a TSK gel-G3000SW column (Tosoh Corporation, Tokyo, Japan) to separate each isoform of VEGF containing 121, 165, 189, and 206 amino acids by molecular weight difference. The supernatant was filtered by Samprep LH4 (0.5 μm; Millipore, Bedford, MA) before high-performance liquid chromatography separation. The sample was injected into the TSK gel-G3000SW column, and the column was eluted with 0.1 M phosphate buffer with 0.2 M sodium chloride, pH 7.2, at a flow rate of 0.5 ml/minute. The VEGF protein concentration of every other fraction sample was measured by enzyme-linked immunosorbant assay. Chymotripsinogen (25 kD; Serva Electrophoresis, Heidelberg, Germany) and egg albumin (45 kD; Serva Electrophoresis) were used as molecular markers. The human recombinant VEGF165 was also eluted under the same conditions, and the VEGF protein concentration of each fraction was measured to assess the fraction profile of VEGF165.

Statistics

The differences between groups were tested for significance using the Kruskal-Wallis test for VEGF comparison. The differences were tested by the values above the detectable limit of VEGF. In all cases, a p value of less than 0.05 was considered significant. Data in figures and tables are expressed as means ± SEM.

RESULTS

BALF Findings in Nonsmoking Volunteers and Normal Smokers

The total cell count in BALF was increased in normal smokers, IPF, PF-CTD, and Sar (Table 2). The percentage of neutrophils was not increased in normal smokers compared with nonsmoking volunteers (Table 2). However, the percentage of neutrophils was increased in IPF and PF-CTD (Table 2). In IPF, PF-CTD, and Sar, there was a decrease in the percentage of macrophages and an increase in lymphocytes (Table 2).

Comparison of Levels of VEGF in Nonsmokers and Normal Smokers

The concentration of VEGF in the BALF of nonsmoking volunteers and normal smokers is shown in Figure 1. The level of VEGF in nonsmoking volunteers was very high. In contrast, in most of the normal smokers, the values of VEGF in BALF were below the detectable limit (Figure 1). The proportion of VEGF isoforms (VEGF121, VEGF165, VEGF185, and VEGF206) was evaluated by high-performance liquid chromatography in three of the nonsmoking volunteers. In all cases, VEGF165 was the only isoform detected.

TABLE 2. CHARACTERISTICS OF BRONCHOALVEOLAR LAVAGE OF THE STUDY SUBJECTS

	Cell Number ($\times 10^5$ cells/ml)	Macrophage (%)	Lymphocyte (%)	Neutrophil (%)	Eosinophil (%)	Albumin Concentration (mg/ml)	VEGF Concentration (pg/ml)
Nonsmokers	0.69 ± 0.15	88.8 ± 1.89	9.9 ± 1.75	1.22 ± 0.55	0.23 ± 0.07	0.098 ± 0.03	256 ± 32
Normal smokers	1.61 ± 0.32*	94.0 ± 1.06*	3.4 ± 0.89	1.85 ± 0.39	1.63 ± 0.97	0.11 ± 0.05	27 ± 12*
IPF	2.21 ± 0.25*	72.0 ± 8.4*	15.2 ± 4.9*	9.6 ± 4.6*	1.83 ± 1.22	0.13 ± 0.01*	62 ± 33*
PF-CTD	2.56 ± 0.38*	72.5 ± 5.0*	13.0 ± 3.0*	13.0 ± 5.1*	1.25 ± 0.97	0.13 ± 0.07*	78 ± 22*
Sar	2.05 ± 0.33*	84.3 ± 3.2*	15.0 ± 3.0*	0.5 ± 0.4	0.22 ± 0.45	0.11 ± 0.08	133 ± 49*

Definition of abbreviations: IPF = idiopathic pulmonary fibrosis; PF-CTD = pulmonary fibrosis associated with a connective tissue disease; Sar = sarcoidosis; VEGF = vascular endothelial growth factor.

* $p < 0.05$ compared with nonsmokers.

Values are mean ± SE.

VEGF Levels in BALF with Pulmonary Fibrosis and Sar

The levels of VEGF in BALF in patients with IPF, PF-CTD, and Sar were significantly less than those in normal nonsmokers (Figure 1, $p < 0.01$). Among these patients, the patients who smoked all had low concentrations of VEGF in BALF (Figure 1).

DISCUSSION

It is reported that the level of VEGF in the epithelial lining fluid was even higher than that in the plasma of healthy volunteers (21). Our results also demonstrate that VEGF protein was present in epithelial lining fluid at high levels in healthy subjects. As determined by Northern blotting on a per actin basis, the order of relative abundance of VEGF mRNA in adult tissue is lung, kidney, liver, brain, and spleen in human, guinea pig, mouse, and rat tissues (22–24), suggesting that the function of VEGF in lung tissue may be critical to normal physiology and, thus, expression is highly conserved. In humans, guinea pigs, mice, and rats (22–24), cellular localization in the lung was observed primarily in the alveolar epithelial cells. Thus, these data support the concept that VEGF is highly compartmentalized in the lung as previously reported (21).

A more complete understanding of the functions of VEGF in the lung depends on several factors: the size and bioactivity of VEGF expressed, the presence of VEGF receptors on adjacent endothelial cells, and the knowledge of mechanisms of both mitogenic and permeability responses. Among them, the size of VEGF secreted is of importance. Although direct analysis of which VEGF forms are expressed has not been systematically performed in the all of the tissues and species in which VEGF has been identified, in human tissues, VEGF165 seems to be the predominant form, followed by VEGF121 and VEGF189 (25, 26). In this study, the isoform of VEGF was VEGF165, and other forms of VEGF were not detected in BALF. Because VEGF165 shows bioactivity and diffusibility to the receptors of endothelial cells (14, 15), VEGF165 in the epithelial lining fluid may regulate many endothelial cell functions.

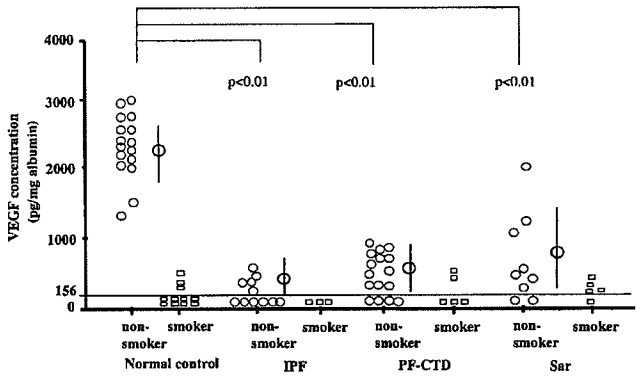

Figure 1. The concentration of VEGF in the BALF of normal volunteers and patients with IPF (n = 14), PF-CTD (n = 21), and Sar (n = 14). *Open circles* represent nonsmokers. *Open squares* represent current smokers. Values are means ± SEM; p values were determined using the data from nonsmokers. Values under the detectable range were estimated at 156 pg/mg albumin for comparison.

VEGF is thought to be involved in the regulation of capillary function, mitogenesis, and permeability-enhancing effects of the lung. However, the finding that expression of VEGF mRNA occurs diffusely in the brain and is concentrated in the cerebellar granule cell layer suggests that neither the angiogenesis nor the permeability-inducing activity of VEGF is of primary importance in the brain. VEGF has been reported to stimulate hexose transport in isolated peripheral endothelial cells (27). VEGF may regulate the transport of small molecules (e.g., glucose and amino acids) to support the increased requirement for energy of epithelial cells. The fact that the decreased level of VEGF not only reduces endothelial cell growth but also induces endothelial cell apoptosis (28) may support mechanisms underlying the decrease in the lung vascular volume observed in pulmonary emphysema. The decreased level of VEGF in normal smokers suggests that the smokers' lungs may have impaired capillary function, mitogenesis, and energy support.

The present authors previously reported that airway epithelial cells express and secrete VEGF165 in response to a variety of stimuli, including interleukin-1β, tumor necrosis factor-α, smoke extract, and neutrophil elastase (29). Thus, it is expected that normal smokers and patients with pulmonary fibrosis would have a higher concentration of VEGF in BALF. However, the lower levels of VEGF in pulmonary fibrosis were consistent with the previous report (30). Although the mechanisms of the decreased levels of VEGF in BALF are uncertain, the epithelial cell apoptosis and cellular injury observed in normal smokers and patients with pulmonary fibrosis (31–33) may be one of the underlying mechanisms that explains the decrease in the release of VEGF. To support this hypothesis, Klekamp and colleagues reported that hyperoxia reduced the level of VEGF in the lungs (34). The reduction of VEGF was correlated with a reduction in the percentage of apoptotic cells.

Another possible explanation for the decreased level of VEGF is due to proteolytic degradation. Proteolytic activity degrades VEGF in a chronic skin wound (35). Whereas plasmin and trypsin both cause VEGF degradation, thrombin, elastase, and collagenase do not cleave VEGF (36). Because smoking and interstitial lung diseases are associated with chronic inflammation in the lungs, it is possible that these protease activities may degrade VEGF, leading to the decreased level of VEGF in BALF. In contrast, Beinert and coworkers reported that oxidant caused upregulation of VEGF in BALF

in patients with lung cancer receiving chemoradiotherapy (37). In their report, increased levels of oxidized methionine indicated that these patients suffered from severe pulmonary oxidative stress, but the VEGF concentration in epithelial lining fluid was significantly elevated, suggesting that its antigeneity is not affected by ambient redox potentials.

Aging is another factor that influences the levels of VEGF in BALF, because BALF VEGF levels decline significantly with advancing age (30). In this study, age was not significantly different among the groups, and thus, age alone cannot explain the reduced level of VEGF in BALF.

In conclusion, VEGF in BALF are significantly reduced in subjects who smoke cigarettes and in patients with IPF, PF-CTD, and Sar. Reduced concentrations of VEGF may be important in the pathogenesis of lung diseases in these patients.

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In Vivo Gene Transfection With Hepatocyte Growth Factor via the Pulmonary Artery Induces Angiogenesis in the Rat Lung

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Background—Recent studies have demonstrated that gene transfer with hepatocyte growth factor (HGF) induces angiogenesis for coronary and peripheral artery diseases. We investigated the ability of gene transfer with human HGF to induce angiogenesis in the rat lung.

Methods and Results—The left lung was selectively transfected with a cDNA encoding human HGF via the left pulmonary artery, using the HVJ-liposome method (H group); rats transfected with the same vector lacking the HGF gene served as controls (C group). HGF gene transfer significantly increased the capillary density in the left lung compared with the C group 7 days after transfection (15.0 ± 1.3 versus 8.0 ± 1.7 mm^2, $P<0.01$). The left to right average blood perfusion ratio detected by laser Doppler imaging increased significantly in the H group 14 days after transfection (1.12 ± 0.09 versus 0.91 ± 0.11, $P<0.01$). A right pulmonary artery clamp test, in which only the left lung received all the pulmonary blood flow from the right ventricle, revealed that the increase in right ventricular pressure was significantly attenuated in the H group compared with the C group 7 days after transfection (8.6 ± 3.5 versus 15.3 ± 2.8 mm Hg, $P<0.01$).

Conclusions—Trans-pulmonary arterial transfer of the human HGF gene into the left lung increased capillary density and blood perfusion, and decreased vascular resistance when blood flow increased. These results suggest therapeutic angiogenesis induced by HGF gene expression in the lung may be found suitable in treating subjects with decreased pulmonary vasculature or increased pulmonary vascular resistance. **(*Circulation.* 2002;106[suppl I]:I-264-I-269.)**

Key Words: angiogenesis ■ gene therapy ■ pulmonary vasculature ■ growth factor ■ remodeling

Although recent advances in cardiovascular surgery have improved clinical results inpatients with decreased pulmonary vasculature or increased pulmonary vascular resistance, such as pulmonary infarction, pulmonary hypertension, or hypoplastic pulmonary vasculature associated with congenital heart disease, the prognosis of critical cases have remained poor.[1–3] A growing body of evidence indicates that in vivo gene transfection with angiogenic factors can induce angiogenesis to treat ischemic coronary and peripheral artery disease;[4,5] this is called therapeutic angiogenesis. If angiogenesis could be achieved for the pulmonary vasculature, the mortality and morbidity of patients with severely decreased pulmonary vasculature could be minimized.

Hepatocyte growth factor (HGF), which was originally purified and cloned as a potent mitogen for hepatocytes,[6] has mitogenic, motogenic, morphogenic, and antiapoptotic activities in various cell types.[7,8] The pluripotent activities of HGF are mediated by a membrane-spanning tyrosine kinase receptor encoded by the *c-met* proto-oncogene.[9] Physiologically, HGF acts as an organotrophic factor for the regeneration and protection of organs, including the liver,[10,11] kidney,[12,13] heart,[14,15] and lung.[16,17] Furthermore, HGF has an angiogenic effect and recent studies have demonstrated that HGF can potently induce angiogenesis to alleviate coronary and peripheral artery disease.[18,19] Clinical studies have begun for the treatment of patients with arteriosclerosis obliterans. HGF's potent angiogenic effect, as well as its other multiple effects, is considered likely to be useful in cases of decreased pulmonary vasculature such as pulmonary infarction, pulmonary hypertension, or hypoplastic pulmonary vasculature of congenital heart disease. However, no study has yet reported angiogenesis in the lung, using in vivo transfection and expression of genes for any angiogenic factor, including HGF. Based on the above background, we hypothesized that in vivo gene transfection of the lung with HGF might possibly become a novel therapy in patients with severely decreased or hypoplastic pulmonary vasculature.

In this study, we determined whether in vivo gene transfection with HGF induces angiogenesis in the rat lung.

Materials and Methods

Animal Care

This study was carried out under the supervision of the Animal Research Committee in accordance with the Guidelines on Animal

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Experiments of Osaka University Medical School and the Japanese Government Animal Protection and Management Law.

Construction of Plasmid With Human HGF gene

To prepare an HGF expression vector, a human HGF cDNA was inserted into the Not I site of the pUC-SRα expression vector plasmid.[20] In this plasmid, expression of the HGF cDNA is regulated under the control of the SRα promoter.[21] We also constructed a control expression vector without the HGF gene.

Preparation of HVJ-Liposome

The preparation of the liposome complexed with the hemagglutination virus of Japan (HVJ) is described elsewhere.[22] Briefly, 10 mg of a lipid mixture (phosphatidylserine, phosphatidylcholine, and cholesterol) was deposited on the side of a flask by removing tetrahydrofuran in a rotary evaporator. The dried lipid was hydrated in 200 μL of a balanced salt solution (137 mmol/L NaCl, 5.4 mmol/L KCl, 10 mmol/L Tris-HCl; pH 7.6) containing a DNA (200 μg)-HMG1 (high mobility group 1 nuclear protein, 64 μg) complex. A liposome-DNA-HMG1 complex suspension was prepared by thorough vortexing, sonicating, and shaking. The liposome suspension was incubated with 30,000 HVJ particles, inactivated by ultraviolet irradiation, first at 4°C and then at 37°C. After a sucrose gradient centrifugation through, 4 mL of the layer containing HVJ-liposomes was collected and used for subsequent experiments.

Surgical Approaches

Wistar rats weighing 170 to 210 g were anesthetized by intraperitoneal injection of 50 mg/kg ketamine (Sankyo Co) and 5 mg/kg xylazine (Bayer Co). The rats were intubated and ventilated with a rodent ventilator (SN480–7, SHINANO Instruments). Breaths were administered at a rate of 80 per minute with a tidal volume of 2 mL.

Gene Transfection of the Left Lung via the Pulmonary Artery

Rats underwent occlusion of the left pulmonary artery and vein with infusion of liposomes into the left lung according to the method of Schachtner et al[23] Briefly, a left thoracotomy was performed by entering the thoracic cavity at the third to fourth intercostal space. The single left lobe was reflected, and the left pulmonary artery and vein were isolated. After arterial inflow was occluded with a small vascular clip, a small arteriotomy was made in the left pulmonary artery distally, and a 28-gauge PI catheter (Argyle) was inserted. The catheter was secured in the left main pulmonary artery with a 3 to 0 silk tie. The left pulmonary vein was occluded with another vascular clip. The HVJ liposome-plasmid complex (0.4 mL, including 20 μg of cDNA) was infused *via* the catheter. After clamping the distal left pulmonary artery with a third vascular clip, the catheter was removed and the arteriotomy was repaired with 9 to 0 sutures. After a 20-minute dwell period, the arterial and the venous clips were removed, and the pulmonary arterial flow was reestablished. The thorax was closed, and the soft tissues and the skin were closed in layers with 3 to 0 silk sutures. The animal was allowed to recover in a warm, oxygenated environment. The expression vector with HGF cDNA was transfected into 40 rats (H group) and the vector without HGF was transfected into another 40 rats, which served as the controls (C group) for the assessment of a pulmonary artery clamp test and histological analysis. Eight rats in each group were sacrificed 4, 7, 14, 21, and 28 days after the transfection, respectively. Another 60 rats (30 rats with HGF and 30 rats with control vector) were transfected for evaluation of pulmonary blood perfusion analysis and ELISA. Six rats in each group were sacrificed 4, 7, 14, 21, and 28 days after transfection, respectively.

Pulmonary Artery Clamp Test

To assess the change in pulmonary vascular resistance after HGF gene transfection, we performed a pulmonary artery clamp test that was designed based on our clinical experience[24] and a preliminary result that rats subjected to a right pulmonary artery clamp tolerated for at least 5 minutes. Briefly, rats were anesthetized, intubated and ventilated again. A small mid-line thoracotomy was carefully performed with an electro-cautery to prevent bleeding. The pulmonary artery trunk was dissected out and taped. The proximal right and left pulmonary arteries were carefully dissected out. The right pulmonary artery was then clamped with a small clip just distal to the bifurcation. The right ventricular systolic pressure before and after clamping was measured by puncture with a 24G needle connected to a transducer (TERUMO) and a polygraph system (Nihon Kohden Co). This measurement was done 3 times, and the increase in right ventricle pressure (ΔRVP) was calculated. After collecting arterial blood from the ascending aorta, the heart and lungs were resected en bloc, and the lungs were cleared of blood by infusing cold phosphate-buffered saline (PBS) through a catheter positioned in the main pulmonary artery. All tissue samples subjected to histological analysis were fixed in ethanol.

Immunohistochemical Analysis

The tissue specimens obtained from the hilum of the left lung were fixed in ethanol, embedded in paraffin, and sectioned. Tissue sections were immunohistochemically stained with a rabbit polyclonal antibody against human HGF, using a standard indirect peroxidase-antiperoxidase method.[25] Briefly, sections with 4-μm-thick were mounted on glass slides coated with 3-aminopropyltriethoxysilane, and air-dried overnight at room temperature. After deparaffinization, endogenous peroxidase was inactivated with methanol containing 0.3% hydrogen peroxidase for 30 minutes. The sections were incubated at 4°C overnight with primary antibodies, and subsequently with biotinylated goat anti-rabbit IgG (DAKO) and biotinylated goat anti-mouse IgG (DAKO) at room temperature for 30 minutes. After incubation with the avidin-biotin-horseradish peroxidase complex (Vector Labs), enzymatic reaction was visualized with DAB, followed by incubation with DAB-enhancing solution (Vector Labs). The sections were counterstained with hematoxylin. Sections were also immunostained with antibodies against factor VIII and PCNA in the same fashion. The number of factor VIII-positive capillaries that were less than 100 μm in diameter was counted under microscopy for 10 randomly selected fields per specimen. The capillary density was determined as the average number of factor VIII-positive capillaries per 1 mm^2. The number of PCNA-positive endothelial cells per 1 mm^2 was also counted in the same fashion.

Laser Doppler Blood Flow Analysis

We measured the left/right lung blood perfusion ratio using a Laser Doppler Image (LDPI) analyzer (Moor Instruments, Cambridge, UK), with modified method used for rat hindlimb ischemia models.[19,26] The blood flow measured by LDPI correlated well with capillary density.[19] In this method, a color-coded image representing blood perfusion distribution is displayed. Low or no blood perfusion is displayed as dark blue, and the highest perfusion level is displayed as red to white. Before measurement, rats were anesthetized, intubated, and connected to a respirator. A midline thoracotomy was performed with an electro-cautery, the chest was opened wide using a set of clamps and the lung surface was exposed. The instrument was then placed above the rat so that the Laser beam scanned the lung surface vertically. After setting the condition of the scan area, ventilation was discontinued, and the lungs were inflated. We performed 2 consecutive LDPI scans over the same region of interest. After the scanning, the average perfusion values of the left (transfected) and right (nontransfected) lungs were calculated from histograms of the colored pixels. To minimize variations because of ambient light, calculated blood perfusion (relative units) was expressed as the left/right lung blood perfusion ratio.[19,26]

Enzyme-Linked Immunosorbent Assay

Human HGF in the left lung was measured by enzyme-linked immunosorbent assay (ELISA), using an anti-human HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan). The human HGF ELISA kit specifically detects human HGF but not rat HGF.

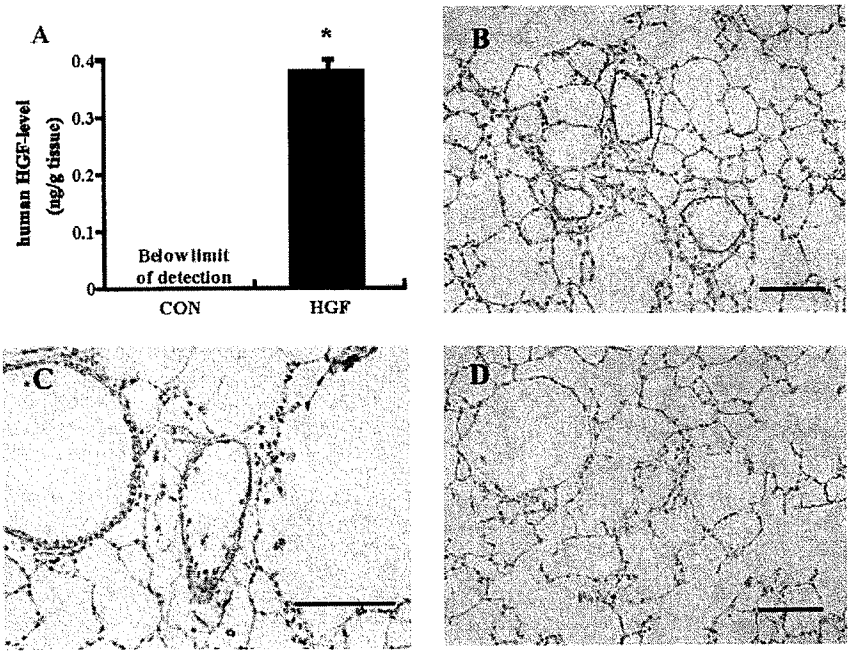

Figure 1. Expression of human HGF in the lung after transfection of HGF gene. (A) Human HGF level in lung tissue on day 4 after gene transfection. Rats were killed on day 4 after gene transfection. Tissue extracts were obtained from rats with either HGF gene transfection (HGF), or mock vector transfection (CON), and HGF levels in tissue extracts were measured using ELISA. Each value represents the mean±SEM of values obtained using 6 rats. *$P<0.01$ versus control (Student's t test). (B–D) Immunohistochemical staining for human HGF in the left lung 4 days after gene transfection. B and C: HGF gene transfected lung; D: control gene transfected lung. Tissue sections were subjected to immunohistochemical staining for the human HGF using an anti-human HGF antibody. Most of endothelial cells of the pulmonary arteries were stained in the lung transfected with HGF gene (B and C), but not in the control lung transfected with mock vector (D). Bar, 100 μm (Magnifications B,D; ×100, C; ×200).

Statistical Analysis

All values are expressed as the mean±SEM. The statistical differences in data obtained by ELISA were determined by a Student's t test. Statistical differences in the other data were assessed by one-way ANOVA followed by Bonferroni's post hoc test. A value of $P<0.05$ was considered to be statistically significant.

Results

Expression of Human HGF Introduced by Gene Transfection

At 4 days after transfection of plasmid, an enzyme immunoassay demonstrated a significant ($P<0.01$) pulmonary expression of human HGF protein in the H group compared with the C group (Figure 1A). Likewise, immunohistochemical examination with an anti-human HGF polyclonal antibody 4 days after transfection showed apparent and extensive expression of human HGF in the cytoplasm of the endothelium of the pulmonary arteries in the H group (Figure 1, B and C). In contrast, human HGF was undetectable in lung tissues in the C group (Figure 1D). These results indicated that human HGF was specifically and predominantly expressed in endothelial cells in the lung transfected with HVJ-liposome containing expression vector for human HGF.

Pathological Evaluation of Angiogenesis by Immunohistochemistry

To evaluate angiogenesis in the lung, we performed an immunohistochemical examination with anti-factor VIII, a specific marker for endothelial cells. At 7 days after transfection, a marked increase in the number of factor VIII-positive pulmonary capillaries was observed in the H group (Figure 2A), compared with the C group (Figure 2B). Change in capillary density determined as Factor VIII-positive capillaries that were less than 100 μm in diameter per square millimeter, indicated that significant increase in capillary vessels was seen on day 4 after gene transfection (Figure 2C). The capillary density reached the maximum level by day 7 and the level was maintained within 28 days. In contrast, the capillary density in control lung transfected mock vector was not changed within 28 days after transfection. On day 7, the capillary density reached 15.0±1.3 in the H group, whereas it remained 8.0±1.7 mm^2 in the C group. Therefore, angiogenesis in the lung was induced after HGF gene transfection, and capillary formation had apparently progressed by 7 days after transfection.

To evaluate endothelial proliferation, proliferating cells were immunohistochemically detected with an anti-PCNA antibody and PCNA-positive endothelial cells per mm^2 was determined. The number of PCNA-positive cells did not change within 28 days in control lung transfected with mock vector (Figure 2D). In contrast, significant and marked increase in the number of PCNA-positive endothelial cells was seen as early as day 4 after transfection of expression vector for HGF. The number of PCNA-positive nuclei in capillaries 7 days after transfection in the H group was 4.8±0.4, whereas in the C group it was 0.8±0.3 mm^2. Thus, the result indicates that HGF gene expression stimulated capillary endothelial proliferation in the lung.

Changes in Pulmonary Vascular Resistance of the Left Lung After HGF Transfection

To assess the hemodynamic change induced by HGF gene transfection, we performed a right pulmonary artery clamp test, in which the left lung received all the pulmonary blood flow from the right ventricle. This test measures changes in the pulmonary vascular resistance of the left lung. The increase in right ventricle pressure after right pulmonary artery clamp (ΔRVP) was 16 mm Hg preoperatively, and it did not change in the C group after transfection. In contrast, a significant attenuation of the ΔRVP was observed in the H group, 7, 14, 21, and 28 days after HGF gene transfection (Figure 3). The ΔRVP value in the H group decreased to

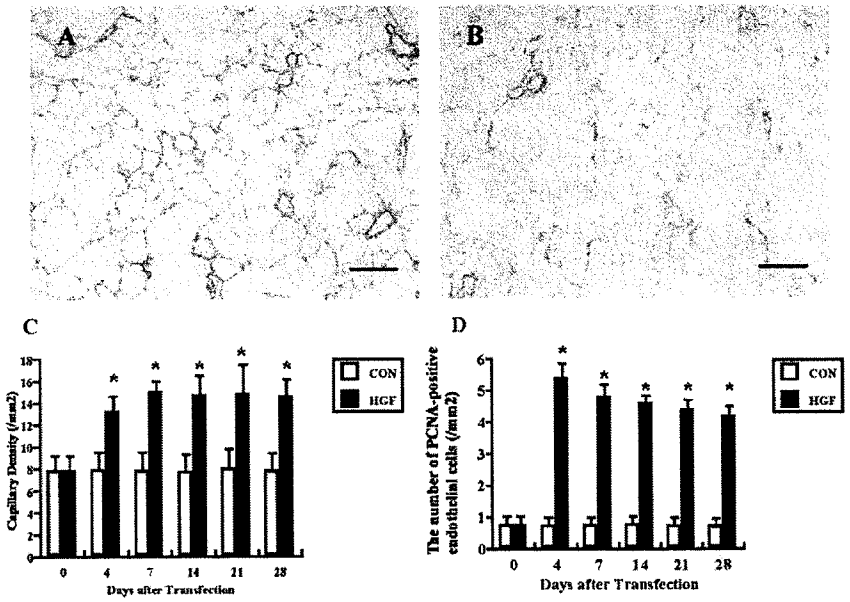

Figure 2. Changes in the number of capillaries and PCNA-positive endothelial cells in the left lung. (A, B) Distribution of capillary vessels in the left lung transfected with HGF gene (A) or mock vector (B) at 7 days after gene transfection. Tissue sections were subjected to immunohistochemical staining using anti-Factor VIII antibody. Bar, 100 μm, (Magnifications A,B; ×100). (C) Change in the number of capillary density. Capillary density was determined as the number of factor VIII-positive capillaries less than 100 μm in diameter per square millimeter. Rats were killed on day 0 (before surgery), 4, 7, 14, 21, and 28 days after gene transfection. Each value represents the mean±SEM of values obtained using 8 rats at each time point in each group. *P<0.01 versus CON (ANOVA). (D) Change in the number of PCNA-positive endothelial cells of pulmonary capillaries in the left lung after transfection of HGF gene (HGF) or control mock vector (CON). Tissue sections were subjected to immunohistochemical staining using an anti-PCNA antibody. Rats were killed on day 0 (before surgery), 4, 7, 14, 21, and 28 days after gene transfection. Each value represents the mean±SEM of values obtained using 8 rats at each time point in each group. *P<0.01 versus CON (ANOVA).

9 mm Hg on day 7, the value was 60% of that in C group, and it remained similar levels within 28 days. Taken together with change in the capillary density induced by HGF gene transfection, these results strongly suggested that angiogenesis induced by HGF gene expression resulted in decreased right ventricle blood pressure after occlusion of right pulmonary artery.

Laser Doppler Analysis for Lung Blood Perfusion

To determine whether blood perfusion increased in the transfected left lung, we measured blood perfusion of the lung surface with a Laser Doppler Imager. Representative images of the lung blood perfusion 14 days after gene transfection are shown in Figure 4A (HGF-transfected) and Figure 4B (control-transfected). The ratio of left (transfected)/right (nontransfected) blood perfusion was 0.95 in both C and H groups before transfection, and it did not change within 28 days after transfection of mock vector. In contrast, the left/right blood perfusion ratio increased significantly 14, 21, and 28 days after transfection of HGF gene (Figure 4C). Thus, average blood perfusion values in the transfected left lung increased significantly by 14 days after the HGF gene transfection.

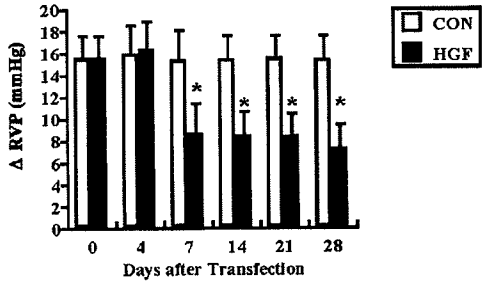

Figure 3. Changes in the pulmonary vascular resistance of the left lung after transfection of HGF (HGF) or control mock vector (CON). The pulmonary vascular resistance was assessed using pulmonary artery clamp test, in which the left lung received all the pulmonary blood flow from the right ventricle. Right ventricle pressure before and after right pulmonary artery clamp was measured and the increase in right ventricle pressure (ΔRVP) was calculated. Rats were killed on day 0 (before surgery), 4, 7, 14, 21, and 28 days after gene transfection. Each value represents the mean±SEM of values obtained using 8 rats at each time point in each group. *P<0.01 versus CON (ANOVA).

Discussion

In this study, we have demonstrated that in vivo transfection of HGF gene *via* the pulmonary artery induced angiogenesis in the lung, and that this angiogenic action might be exerted through the direct action of HGF on the endothelial cells of pulmonary capillaries. This conclusion is supported by the following evidence: (1) human HGF was expressed predominantly in the cytoplasm of endothelial cells; (2) the number of factor VIII-positive pulmonary capillaries significantly increased; (3) the blood perfusion of the left lung surface, as assessed by the Laser Doppler Imager, increased; and (4) left pulmonary vascular resistance, assessed by the pulmonary artery clamp test, decreased after HGF gene transfection. We previously demonstrated that in vivo tansfection of HGF gene induced angiogenesis in the ischemic hindlimb[19] and the infarcted heart.[14,18] However, our present results are the first to show that in vivo transfection of HGF gene induces angiogenesis in the lung.

We evaluated capillary blood vessels in the lung after transfection and expression of HGF gene, both histologically and functionally. There was no histological abnormality in vascular structure such as pulmonary arterio-venous fistula[29] and edema-like pathology because of immature vascularity by the experts of lung pathology. No significant decrease was seen in oxygen saturation in arterial blood after expression of HGF gene (data not shown). We could not see any general side effects during and after gene expression of HGF. Likewise, previous approaches on therapeutic angiogenesis with the similar delivery method of HGF gene in hind-limb

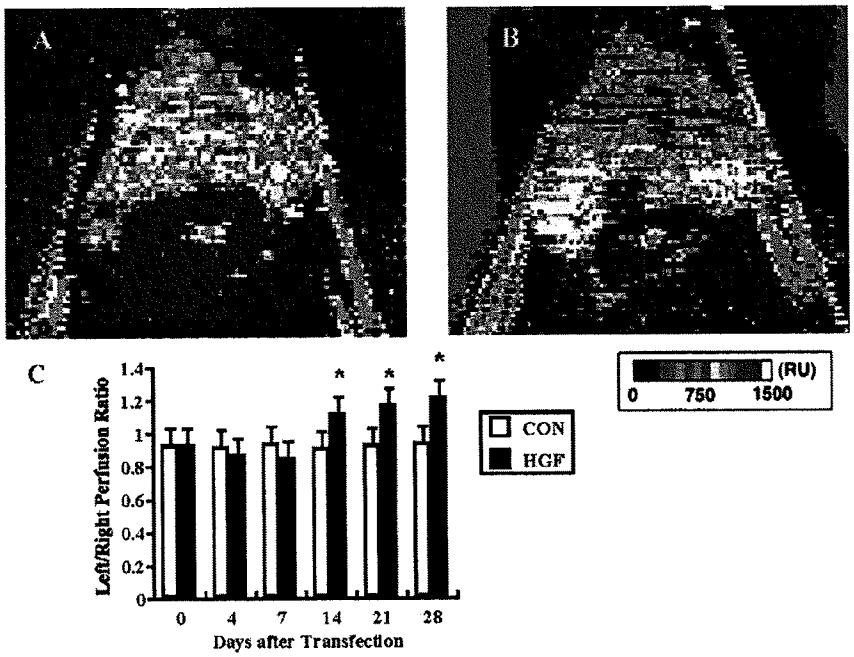

Figure 4. Changes in the blood perfusion of the left lung after gene transfection. Blood perfusion of the lung was assessed using Laser Doppler Imager. (A, B) Representative Laser Doppler Image analysis of lung blood perfusion at 14 days after transfection of HGF gene (A) or mock vector (B). RU indicates relative units. (C) Change in the Left/right lung blood perfusion ratio after transfection of HGF gene (HGF) or control mock vector (CON). Left/right lung blood perfusion ratio was calculated using the average values of blood perfusion in the left (transfected) and the right (nontransfected) lung. Rats were measured on day 0 (before surgery), 4, 7, 14, 21, and 28 days after gene transfection. Each value represents the mean±SEM of values obtained using 6 rats at each time point in each group. *$P<0.01$ versus CON (ANOVA).

and ischemic heart models did not note abnormality in vascular structures and functions, and general side effects during the treatment. In stead, aberrant expression in c-Met receptor and HGF genes were noted in patients with lung cancer.[27,28] However, neoplastic changes in various tissues of rats treated with HGF gene transfer were not seen in the present study, as well as previous studies on HGF gene therapy in experimental animals.[18,19] Because HVJ-liposome-mediated HGF gene transfection allows local and transient but not persistent expression of HGF gene, and it did not allow systemic increase in blood HGF levels (not shown). HVJ-liposome-mediated HGF gene expression is likely to be safe and distinguished from aberrant expression and genetical alteration in c-Met receptor and HGF genes in cancer tissues. Nevertheless, because HGF enhances invasion of cancer cells, including lung carcinoma cells,[27,28] careful analysis to evaluate potential involvement of HGF gene transfer in regulation of invasion and angiogenesis in lung cancer remains to be addressed.

Most recent studies of in vivo gene transfer to the lung used the intra-tracheal method of gene transfection.[30–32] This method is technically easier than the trans-pulmonary arterial approach, but the transfected gene is expressed mainly in the alveolar epithelial cells or pneumocytes, and endothelial expression is difficult to obtain. In contrast, because the purpose of this study was to induce angiogenesis of the pulmonary arteries, we chose the method of transpulmonary arterial gene transfection. Our result showed the arterial transfection of HGF gene resulted in predominant expression in the pulmonary endothelial cells and proved our original hypothesis that the preferential expression of HGF gene in endothelial cells of the pulmonary arteries would sufficiently induce endothelial proliferation and subsequent vascular formation. In regard to technical details, Jeppesson et al[33] first showed the efficacy of the trans-pulmonary venous approach in the lung transplantation model. However, their technique increased perfusion of the bronchial circulation, which was deleterious for the induction of pulmonary arterial angiogenesis. We used a 20-minute dwell time to permit liposome contact with endothelial cells. Mechanical ventilation may help improve the transfer efficiency.

HGF is a potent angiogenic factor, and could be useful as a clinical agent.[18,19] We believe that promoting the endogenous angiogenic system through genetic engineering is a promising advanced strategy for regenerating the pulmonary vasculature. One of the advantages of gene transfer over recombinant protein administration is a persistent, localized delivery of the protein.[34] Systemic delivery of recombinant protein has limitations that include the necessity of repeated injections of a large amount of peptide with possible side effects in other organs. Local drug delivery also has to be repeated with special equipment to sustain a high level of HGF in the lung. In contrast, gene transfection could effectively express the protein in the lung for the necessary time without repeated treatment. Thus, enhancing pulmonary HGF expression through gene transfection could be a promising therapeutic strategy.

Our strategy of gene therapy with angiogenic factors for the treatment of the lung is intended to combine with surgery. From a clinical perspective, the trans-pulmonary approach is feasible when it is combined with a conventional surgical procedure such as endoarterectomy or a systemic-pulmonary shunt. This combined therapy may increase both pulmonary blood flow and the pulmonary vascular bed. This is important because even the gene expression obtained by transfection is transient. The safety of the HVJ liposome method has been reported,[35] and a clinical trial of therapeutic angiogenesis with HGF gene for peripheral artery disease has begun with good short-term results. Therefore, our method may become a novel strategy for patients with severely decreased pulmonary vasculature.

The main limitation of this study is that our data were obtained using the normal rat lung. There are some differ-

ences in the condition of pulmonary vasculature in normal and diseased lungs, including the tissue level of angiogenic factors and endothelial function. Further investigation is required under conditions that represent the damaged or underdeveloped lung, including pulmonary infarction or pulmonary hypertension models, to assess the potential response of the damaged endothelium to HGF and to assess its other effects in the damaged pulmonary vasculature.

In summary, in vivo gene transfection of the lung with HGF by means of the pulmonary arterial injection of HVJ-liposomes caused pulmonary vascular endothelial-cell proliferation, ameliorated the increase in pulmonary vascular resistance when pulmonary blood flow increased, and increased pulmonary blood flow in the left lung, suggesting pulmonary angiogenesis. These data indicate a direct role for HGF in angiogenesis in the lung, suggesting the possibility that gene transfection with HGF may become a novel gene therapy for patients with severely decreased pulmonary vasculature.

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Cell-Based Gene Transfer of Vascular Endothelial Growth Factor Attenuates Monocrotaline-Induced Pulmonary Hypertension

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Cell-Based Gene Transfer of Vascular Endothelial Growth Factor Attenuates Monocrotaline-Induced Pulmonary Hypertension

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Background—Pulmonary arterial hypertension is characterized by increased pulmonary vascular resistance secondary to a decrease in the caliber and number of pulmonary vascular channels. We hypothesized that the targeted overexpression of an angiogenic factor within the lung would potentially minimize the development and progression of pulmonary arterial hypertension by preventing the loss of existing vessels or by inducing the development of new blood vessels within the lung.

Methods and Results—We used a cell-based method of gene transfer to the pulmonary microvasculature by delivering syngeneic smooth muscle cells overexpressing vascular endothelial growth factor (VEGF)-A to inbred Fisher 344 rats in which pulmonary hypertension was induced with the pulmonary endothelial toxin monocrotaline. Four weeks after simultaneous endothelial injury and cell-based gene transfer, right ventricular (RV) hypertension and RV and vascular hypertrophy were significantly decreased in the VEGF-treated animals. Four weeks after gene transfer, the plasmid VEGF transcript was still detectable in the pulmonary tissue of animals injected with VEGF-transfected cells, demonstrating survival of the transfected cells and persistent transgene expression. In addition, delay of cell-based gene transfer until after the development of pulmonary hypertension also resulted in a significant decrease in the progression of RV hypertension and hypertrophy.

Conclusions—These results indicate that cell-based VEGF gene transfer is an effective method of preventing the development and progression of pulmonary hypertension in the monocrotaline model and suggest a potential therapeutic role for angiogenic factors in the therapy of this devastating disease. **(*Circulation*. 2001;104:2242-2248.)**

Key Words: angiogenesis ■ gene therapy ■ endothelium-derived factors ■ hypertension, pulmonary ■ remodeling

Primary arterial hypertension is a progressive disease of unknown pathogenesis with few satisfactory long-term treatment options.[1] The majority of patients with primary pulmonary hypertension (PPH) show little or no acute vasodilator response, and their prognosis remains grave, with a mean survival of ≈3 years from the time of diagnosis.[2] The introduction of intravenous prostacyclin analogues has represented a significant advance in the treatment of this disease; the long-term benefits have not been established, however, and the treatment itself is associated with significant morbidity.[3,4] Endothelin receptor antagonists have been found to have favorable hemodynamic effects in patients with severe congestive heart failure and associated PH,[5] although the experience in patients with primary arterial hypertension is limited. Similarly, the administration of NO has met with some clinical success,[6,7] but difficulties associated with long-term inhalational delivery and concerns over its safety and efficacy have limited widespread application.[8]

Recently, efforts to identify the potential factors underlying PPH have focused on the involvement of growth factors.[9] Upregulation of vascular endothelial growth factor (VEGF) has been described in association with plexiform lesions, possibly representing an incomplete attempt at revascularization distal to arteriolar occlusion.[10] In the monocrotaline (MCT) model of PH, however, an overall decrease in pulmonary VEGF expression has been reported,[11,12] in concert with a dramatic decrease in pulmonary vessel number and a significant increase in vessel wall thickness.[12] Therefore, we hypothesized that the overexpression of VEGF within the pulmonary microvasculature would reduce the development of PH in the MCT model of disease. To deliver VEGF to the lung, we exploited the natural filtering properties of the pulmonary microvasculature by using a novel cell-based pulmonary gene transfer approach.[13]

We now report that cell-based gene transfer of VEGF reduced the development of MCT-induced PH in the rat and decreased the hypertrophic response seen in the right ventricle (RV) and pulmonary vasculature in this model. These results suggest that the delivery of vascular endothelial

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growth factor is an effective method of preventing PH and underscore the importance of VEGF in the maintenance of pulmonary vascular homeostasis.

Methods

Cell-Based Pulmonary Vascular Delivery and Localization

All animal experiments were performed in accordance with the Canadian Council on Animal Care guidelines. Fisher 344 rats (Charles River Co, St Constant, Quebec) were obtained at 21 days of age and euthanized. The main pulmonary arteries were excised and smooth muscle cells cultured.[14] Cells between the fifth and ninth passages were labeled with the viable fluorophore chloromethyl trimethyl rhodamine (CMTMR, Molecular Probes Inc) and were then injected into the internal jugular vein of recipient Fisher 344 rats. Animals were allowed to survive for 1 minute, 30 minutes, 15 hours, and 24 hours (n=3 per group) and were then euthanized. Lung sections were cut, washed with PBS, fixed in acetone-methanol (1:1) for 10 minutes, and incubated for 1 hour with a rabbit-derived polyclonal anti–von Willebrand factor antibody (Sigma Chemical Co) diluted 1:50 in a PBS solution containing 5% horse serum, 5% FCS, and 0.1% Triton X-100. The sections were incubated for 1 hour with goat anti-rabbit FITC-conjugated IgG (Vector Laboratories) diluted 1:100 in the above buffer. Negative control slides were incubated with the same solutions with the primary antibody omitted. The sections were mounted on glass slides with Vectashield (Vector Laboratories) and examined with a scanning confocal microscope (MRC-600, BioRad). The number of cells present within each lung section and their localization with regard to the endothelial border were assessed.

MCT Experiments

The full-length coding sequence of $VEGF_{165}$ was generated by reverse transcription–polymerase chain reaction (RT-PCR) using total RNA extracted from human aortic smooth muscle cells and the following sequence-specific primers: sense, 5′-TCGGGCCTCCGAAACCATGA-3′; antisense, 5′-CCTGGTGAGAGATCTGGTTC-3′. This generated a 649-bp fragment that was sequenced and cloned into the expression vector pcDNA 3.1 (Invitrogen) at the *Eco*R1 restriction site, and correct orientation was determined by use of a differential digest. The insert-deficient vector (pcDNA 3.1) was used as a control for the MCT experiments. Smooth muscle cells were transfected by use of Superfect (Qiagen Inc) with either pcDNA 3.1 or pVEGF and were then trypsinized and divided into aliquots of 500 000 cells. Six- to 8-week-old Fisher 344 rats were injected with saline to establish normal hemodynamic and morphometric parameters (n=7). Experimental animals were injected with 80 mg/kg MCT SC (Aldrich Chemical Co) either alone (n=9) or together with 500 000 pVEGF-transfected (n=11) or pcDNA 3.1-transfected (n=10) cells delivered via a catheter in the external jugular vein. At 28 days after injection, the animals were reanesthetized, and RV systolic pressure (RVSP) and systemic arterial pressure (SAP) were recorded with a Millar microtip catheter inserted into the RV ascending aorta. Before the catheter was placed into the aorta, 0.5 mL of arterial blood was drawn into a heparinized syringe and immediately analyzed for pH, P_{CO_2}, P_{O_2}, and oxygen saturation with a blood gas analyzer. The animals were then killed and the hearts excised. The RV to left ventricular plus septal (LV) weight ratios (RV/LV ratio) were determined as an indicator of hypertrophic response to long-standing PH. Lungs were flushed via the pulmonary artery and gently insufflated with 2% paraformaldehyde via the trachea. The RV systolic pressures and RV/LV ratios were compared between the pVEGF, pcDNA 3.1, and MCT-alone groups.

To determine the effect of cell-based gene transfer of $VEGF_{165}$ on established pulmonary hypertension, 6- to 8-week-old Fisher 344 rats were injected subcutaneously with 80 mg/kg of MCT. Fourteen days after MCT injection, the animals were anesthetized, a Millar catheter was passed into the RV, and the RV pressure was recorded. Pulmonary artery smooth muscle cells transfected with either pVEGF (n=10) or pcDNA 3.1 (n=8) were then injected in aliquots of 500 000 cells into the external jugular vein, and the animals were allowed to recover. At 28 days after MCT injection (14 days after gene transfer), the animals were reanesthetized, and RVSP, SAP, and RV/LV ratios were determined as above.

VEGF RT-PCR

RNA extracted from rat lungs was quantified, 5 μg of total RNA from each animal was reverse-transcribed, and an aliquot of the resulting cDNA was amplified by PCR using the following sequence-specific primers: sense, 5′-CGCTACTGGCTTATCGAAATTAATACGACTCAC-3′; antisense, 5′-GGCCTTGGTGAGGTTTGATCCGCATAAT-3′, for 30 cycles with an annealing temperature of 65°C. The upstream primer was located within the T7 priming site of the pcDNA 3.1 vector, and the downstream primer was located within exon 4 of the coding region of VEGF, thus selectively amplifying a 480-bp fragment only in the presence of exogenous pVEGF mRNA. To amplify the total cellular VEGF transcript, ie, both exogenous and endogenous, a second aliquot of the same reverse-transcription reaction was amplified with the following primers: sense (located within the 5′ UTR of the VEGF transcript), 5′-TCGGGCCTCCGAAACCATGA-3′; antisense (located within exon 8), 5′-CCGCCTCGGCTTGTCACATCT-3′, for 32 cycles with an annealing temperature of 62°C, generating a 589-bp fragment. Finally, a third aliquot of the same reverse-transcription reaction was amplified with the following primers for the constitutively expressed gene GAPDH: sense, 5′-CTCTAAGGCTGTGGGCAAGGTCAT-3′; antisense, 5′-GAGATCCACCACCCTGTTGCTGTA-3′. This reaction was carried out for 25 cycles with an annealing temperature of 58°C. In all cases, 10 μL of a 50-μL reaction were run on a 1.5% agarose gels.

Morphometric Analysis

Paraformaldehyde-fixed rat lungs were paraffin-embedded en face. Sections 5 μm thick were cut and stained by the elastin–van Gieson's technique. A blinded observer measured all vessels with a perceptible media within each cross section under ×40 magnification using the C-Imaging morphometric software (Compix Inc). The medial area of each vessel was determined, and an average was obtained for each vessel size from 0 to 30 and 30 to 60 μm in external diameter for each animal. The averages from each size were compared between the VEGF, pcDNA 3.1, and MCT-alone groups. Similar measurements were made in the experiments in which VEGF gene transfer was given 2 weeks after MCT (ie, delayed gene transfer), except that the images were collected with a Cool SNAP high-resolution CCD camera (Roper Scientific) and analyzed with Scion Image (Scion Corp) morphometric software. In addition, in the delayed gene transfer group, only the VEGF and MCT-alone groups were compared.

Caspase-3 Immunostaining

Lungs were harvested from rats treated with MCT alone or together with VEGF-transfected cells at 1, 2, 3, and 4 weeks. Formaldehyde-fixed sections were cut and mounted, and immunohistochemistry was performed with an antibody for active caspase-3 (Promega).

Statistical Analysis

Data are presented as mean±SD unless otherwise stated. Differences in the number of fluorescently labeled cells over time were assessed by ANOVA, with a post hoc analysis using the Bonferroni test. All pressures, weights, arterial blood gas results, RV/LV ratios, and morphometric data were initially analyzed to determine whether the assumptions for parametric testing (normal distributions and equal variances) had been met. Because these assumptions were met for the pressure, weight, arterial blood gas, and RV/LV ratio data, differences were assessed by ANOVA, with a post hoc analysis using the Bonferroni test. For the morphometric data, the assumption of normal distribution was not met for the 30- to 60-μm vessel grouping. Therefore, a resampling procedure was used to test for differences between the VEGF, pcDNA 3.1, and MCT groups in both the 0- to 30- and 30- to 60-μm groups using sampling with replacement (bootstrapping).[15] The resampling procedure was repeated 2000 times. In all instances, a value of $P<0.05$ was accepted to denote statistical significance. For the morphometric data in the

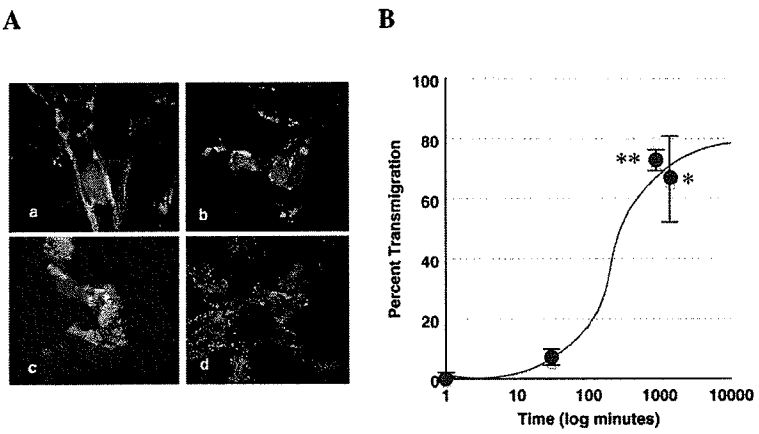

Figure 1. A, Detection of CMTMR-labeled smooth muscle cells in lung sections at various times after internal jugular injection: a, 5 minutes; b, 15 minutes; c, 30 minutes; and d, 15 hours. B, Percentage of CMTMR-labeled cells clearly identified outside microvascular lumen at various time points after CMTMR-labeled cell delivery at 5 and 30 minutes and 15 and 24 hours, n=3 for each time point. *$P<0.05$, **$P<0.001$ vs 5 minutes.

delayed gene transfer experiments, because only 2 groups were being compared, a Student's t test was used for statistical analysis.

Results

Fluorescent Cell Labeling and In Vivo Detection of Labeled Cells

In vitro studies demonstrated that 100% of cultured rat pulmonary artery smooth muscle cells fluoresced intensely after incubation with the fluorophore CMTMR. Five minutes after intravenous injection, nearly all of the CMTMR-labeled cells (99±1%) were found within small arterioles, with little change up to 30 minutes (93±5%, P=NS) (Figure 1). In contrast, by 15 and 24 hours after injection, the majority of CMTMR-labeled cells could be clearly identified outside the endothelial perimeter (73±5% and 67±16%, respectively, $P<0.05$ versus 5 minutes). No fluorescent signals were seen in lungs injected with nonlabeled smooth muscle cells.

MCT Experiments

Four weeks after injection of MCT alone, RVSP was increased from 22±4 mm Hg in normal rats to 49±6 mm Hg, consistent with the development of PH ($P<0.001$, Figure 2A). There was no improvement in animals receiving cells transfected with the control pcDNA 3.1 vector, with the average RVSP remaining at 48±6 mm Hg. In contrast, in animals treated with the pVEGF-transfected cells, RVSP was reduced to 32±7 mm Hg ($P<0.001$ versus MCT alone or null vector). This treatment, however, did not completely restore the RVSP to normal values ($P<0.05$ versus normal animals).

MCT-treated animals receiving pVEGF-transfected cells exhibited better general appearance and weight gain compared with animals treated with either MCT alone or MCT in combination with the delivery of cells transfected with the null pcDNA 3.1 vector (weight increase: 71±80 versus 3±53 and 4±37 g, respectively, $P<0.01$). Arterial oxygen tension (Po_2) was significantly reduced in the MCT-treated rats compared with control animals (Po_2, 53±16 versus 81±9 mm Hg, respectively, $P<0.01$), and delivery of control vector– or VEGF-transfected cells did not result in further worsening of pulmonary gas exchange (Po_2, 65±2 and 64±6 mm Hg, respectively, P=NS versus MCT alone).

Consistent with long-standing and severe PH, the RV/LV ratio was significantly elevated in animals treated with MCT alone (0.34±0.05) or MCT together with pcDNA 3.1–transfected cells (0.33±0.05) compared with normal animals (0.21±0.02, $P<0.001$) (Figure 2B). In contrast, in the group receiving VEGF-transfected cells, the RV/LV ratio was reduced to values not significantly different from those of the control animals (0.24±0.04, $P<0.001$ versus pcDNA or MCT, $P>0.05$ versus control).

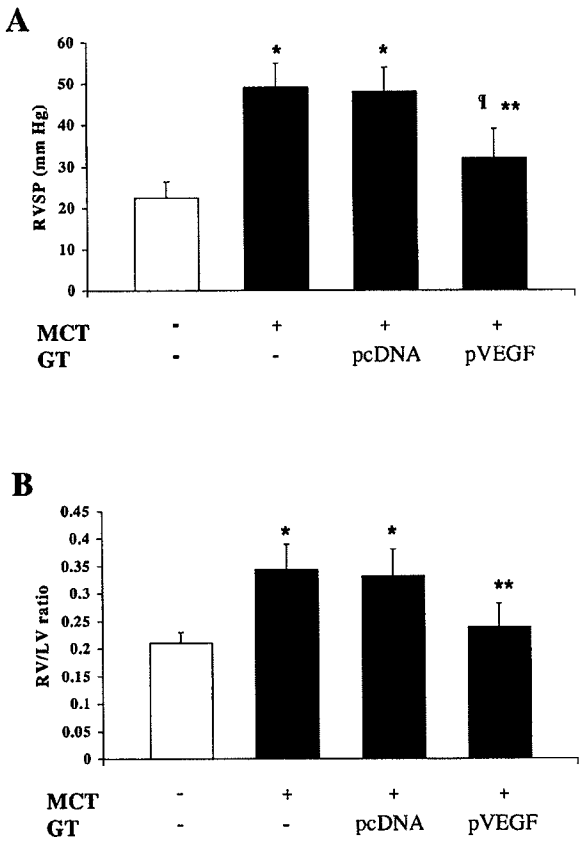

Figure 2. A, RVSP for normal animals (n=7) and for animals 4 weeks after injection of pulmonary endothelial toxin MCT given either alone (n=9) or together with cell-based gene therapy (GT) with null vector–transfected (pcDNA, n=10) or VEGF-transfected (VEGF, n=11) smooth muscle. B, RV/LV ratio for same groups. *$P<0.001$, ¶$P<0.05$ vs control; **$P<0.001$ vs MCT and pcDNA3.1.

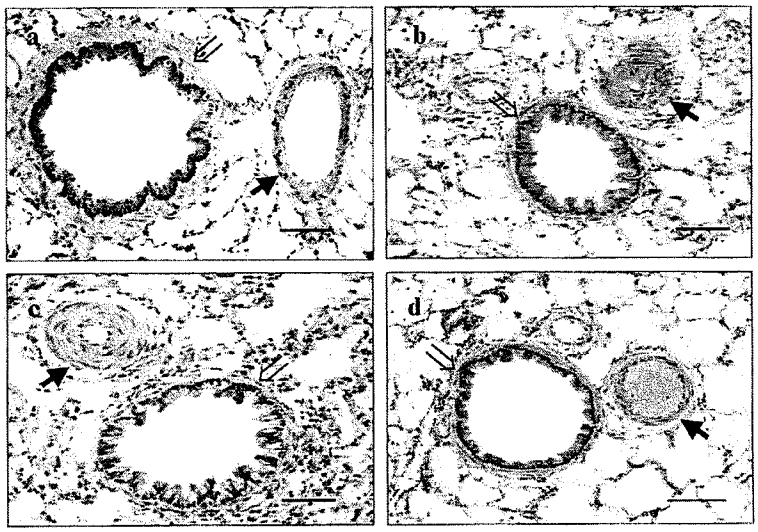

Figure 3. Compared with lungs from normal rats (a), there was marked medial hypertrophic and hyperplastic response in medium and small muscular pulmonary arteries 4 weeks after subcutaneous injection of pulmonary endothelial toxin MCT (b), as well as increased muscularization of distal arterioles. MCT-treated animals treated with pcDNA-transfected cells (c) had similar appearance. In contrast, after cell-based gene transfer of VEGF (d), a decrease in medial thickness was observed in medium and small vessels, with better preservation of overall alveolar structure. Bar=50 μm. Solid arrows indicate muscular arterioles; open arrows, bronchioles.

Figure 3 shows representative sections of lung tissue from normal rats and rats treated with MCT alone or together with cell-based gene transfer of the null or VEGF vectors. In both the MCT-alone and null-transfected animals, exuberant hypertrophy of the media of small muscular arteries and arterioles, as well as increased muscularity of the terminal arterioles, was apparent. As in previous reports using the MCT model,[16,17] morphometric analysis of the pulmonary tissue sections revealed that MCT significantly increased the medial area of small muscular arteries and arterioles compared with rats that were not treated with the pulmonary endothelial toxin (Figure 4). Medial area in the MCT-alone group was not different from the group receiving MCT together with pcDNA 3.1–transfected cells. Cell-based gene transfer with VEGF, however, significantly reduced the medial area measurement in arterioles <30 μm (Figure 4A) and between 30 and 60 μm (Figure 4B) in diameter, compared with either the pcDNA or MCT groups ($P<0.005$).

PCR Analysis

At the time of death, rats treated with MCT alone or MCT and cells transfected with the control vector demonstrated only weak endogenous VEGF expression. In contrast, rats that received the VEGF-transfected cells had a significant increase in total VEGF levels (Figure 5). By use of primers designed to amplify exclusively the plasmid-derived human VEGF transcript, an exogenous VEGF transgene could be detected only in those animals that received the VEGF-transfected cells, confirming that the VEGF transgene expression persisted even 28 days after cell-based gene transfer. In the majority of these animals, no VEGF transgene could be detected in tissues other than lung. A weak band was detected occasionally (3 of 13 experiments, <10%), however, in the spleen and liver. The intestine and kidney did not exhibit any evidence of transgene expression in any animal studied.

Delayed VEGF Gene Transfer

RVSP was elevated to 27±1 mm Hg 2 weeks after MCT injection. In animals receiving pcDNA 3.1–transfected cells at the 2-week time point, the pressure was further increased to 57±14 mm Hg at 4 weeks after MCT delivery. In the pVEGF-treated animals, however (Figure 6), the RVSP increased only modestly (+11±9 mm Hg, $P<0.05$, to 37±7 mm Hg, $P<0.01$ versus pcDNA 3.1) (Figure 6A). The

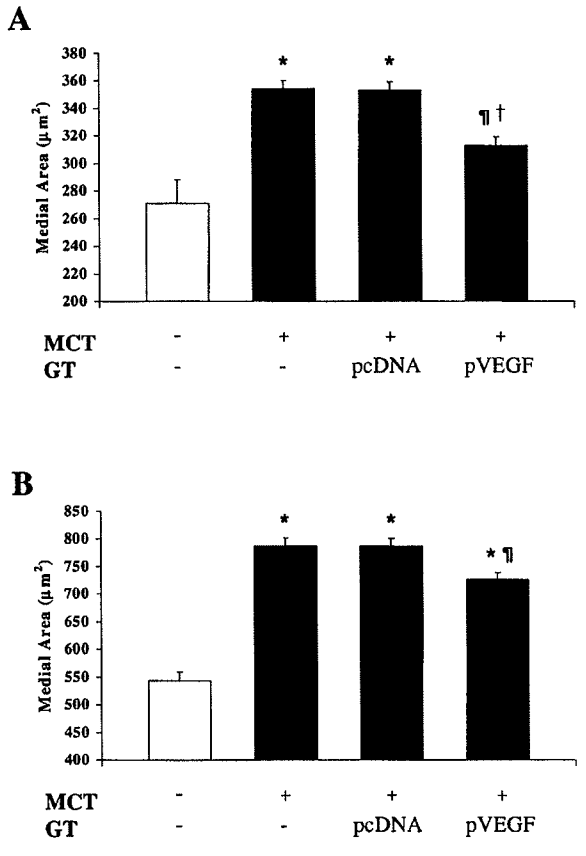

Figure 4. Summary data for medial area of pulmonary arterial vessels <30 μm (A) and 30 to 60 μm (B) in external diameter. Compared with normal control lungs (n=3), lungs from animals treated with MCT alone (n=12) or MCT with pcDNA (n=9) exhibited marked increase in medial area. Treatment with pVEGF-transfected smooth muscle cells (n=11) substantially reduced medial area in MCT-treated animals, particularly in small arterioles (A). Data are mean±SEM. *$P<0.001$, †$P<0.05$ vs control; ¶$P<0.005$ vs MCT or pcDNA.

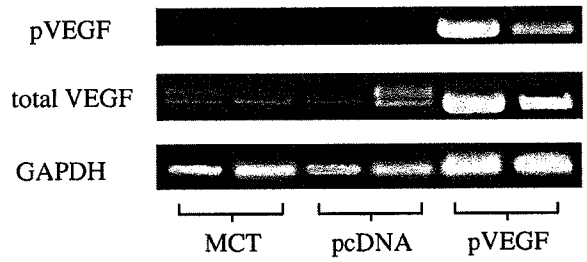

Figure 5. With plasmid-based primers (top), exogenous VEGF transcript was amplified selectively by PCR. In animals injected with VEGF-transfected cells, a consistent signal could be detected corresponding to expected size (lanes 5 and 6); however, no signal was detectable in either MCT-alone– (lanes 1 and 2) or pcDNA-transfected cells (lanes 3 and 4). With primers designed to amplify both VEGF transgene and endogenous VEGF message, a weak signal was detected in lungs from animals treated with MCT alone or MCT and pcDNA-transfected cells, with occasional double bands being detected, indicating amplification of both 121 and 165 isoforms of VEGF generated by alternate splicing. A much stronger signal could be seen in VEGF-transfected animals. RNA quality and loading were assessed by amplification of housekeeping gene GAPDH.

RV/LV ratio was elevated in the pcDNA group to 0.395±0.063, but after VEGF gene transfer, the ratio was reduced to 0.278±0.036 ($P<0.0005$ versus pcDNA 3.1). Again, no difference in aortic blood pressure was noted. If anything, the reduction in medial area was more pronounced after delayed VEGF gene transfer for both the smaller (<30-μm) and larger (30- to 60-μm) arterioles (Figure 6B).

Caspase-3 Immunostaining

Apoptosis of vascular cells was studied at 1, 2, 3, and 4 weeks after MCT treatment alone or together with cell-based VEGF gene transfer (n=2 to 3 for each group). In lungs from animals given MCT alone, staining for active caspase-3 was observed, particularly localized to endothelial cells in the smaller arterioles, and was greatest at 2 weeks (Figure 7A), whereas smooth muscle cells and pericytes only infrequently exhibited caspase-3 positivity. In animals treated with VEGF together with MCT, there was noticeably less endothelial caspase-3 staining (Figure 7B), suggesting that the improvement in pulmonary hemodynamics and arteriolar remodeling induced by VEGF may be associated with reduced endothelial cell apoptosis.

Discussion

Cell-based gene transfer was associated with a high percentage of cells being retained within the lung immediately after intrajugular injection,[13] and the majority of these cells transmigrated through the endothelial layer within 24 hours. The targeting of VEGF-transfected cells to the precapillary resistance vessel reduced the development of MCT-induced PH and inhibited pulmonary vascular and RV remodeling. We have previously shown that cell-based eNOS gene transfer to the pulmonary microvasculature also attenuated the chronic increase in pulmonary arterial pressures in the MCT model of pulmonary vascular remodeling and PH.[13] This approach may offer significant advantages over other strategies to achieve selective pulmonary gene transfer,[18] such as endotracheal

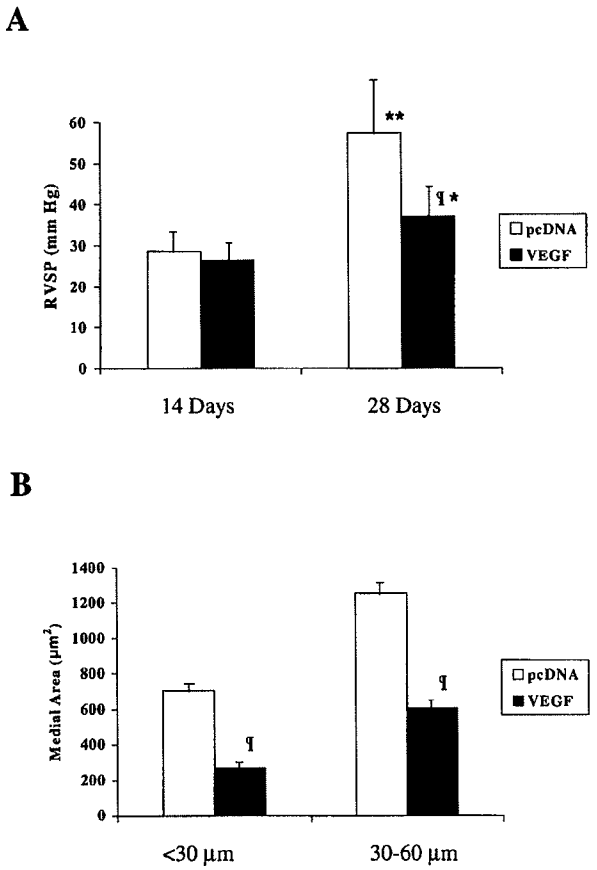

Figure 6. RVSP at 14 and 28 days after MCT injection (A). At 14 days, pressures were determined before delivery of null-transfected (pcDNA) (n=5) or VEGF-transfected (n=7) cells. Pressures were remeasured at 28 days (2 weeks after cell-based gene therapy) in both groups. B, Medial area of pulmonary arterial vessels <30 μm and 30 to 60 μm in external diameter at 28 days for lungs from animals treated with MCT and pcDNA-transfected (n=3) or pVEGF-transfected (n=4) smooth muscle cells. *$P<0.05$ vs 14 days; **$P<0.01$ vs 14 days; ¶$P<0.01$ vs pcDNA. Data for medial area are mean±SEM.

gene delivery, which results in predominantly epithelial overexpression, or catheter-based pulmonary vascular gene transfer, which produces diffuse macrovascular and systemic overexpression.[19]

VEGF in Pulmonary Hypertension

To the best of our knowledge, this is the first report demonstrating that an angiogenic growth factor, such as VEGF, might have therapeutic effects in MCT-induced PH. Increased VEGF expression has been reported in association with plexiform lesions in pulmonary tissue from patients affected by PPH[20]; this was interpreted, however, as suggesting a role for VEGF in the pathogenesis of this disease. Against this view are the results demonstrating potentiation of hypoxic PH with selective blockade of the VEGFR-2 (*kdr*) receptor.[21] Together with the findings of the present study, these data suggest that VEGF expression may represent an adaptive response to microvascular obstruction in PH[10] and that further overexpression achieved by gene transfer may reduce adverse vascular remodeling.

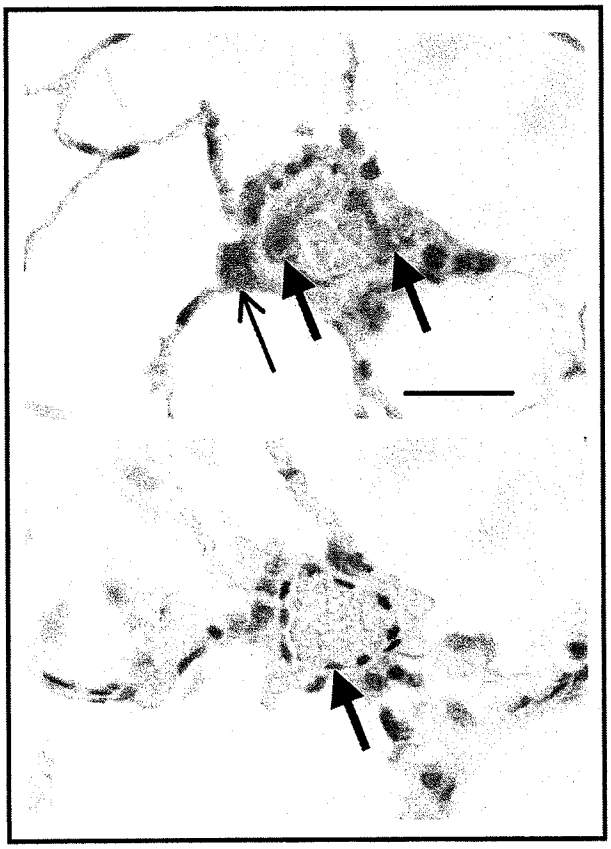

Figure 7. Representative examples of active caspase-3 immunostaining of lung sections harvested 2 weeks after MCT alone or together with cell-based gene transfer of VEGF. In animals treated with MCT alone (top), immunostaining was seen largely localized to endothelial cells of small arterioles (thick arrows), with occasional positivity of surrounding pericytes (thin arrow), whereas in sections from animals treated with MCT and VEGF gene transfer (bottom), endothelial staining for active caspase-3 was infrequent.

The present research did not attempt to specifically address the mechanism of benefit of VEGF gene transfer in the MCT model of PH. VEGF is capable of stimulating NO release from vascular endothelium and increasing local eNOS expression.[22,23] Therefore, it is possible that increased NO production may contribute significantly to the observed benefits in this study. Indeed, it has recently been recognized that NO is a downstream mediator of many of the cellular actions of VEGF,[24] as well as other angiogenic growth factors.[25] It is likely, however, that the direct actions of VEGF, including those on endothelial cell growth and survival, also contributed to the reduction in development of PH after MCT injection. MCT exposure has been shown to result in a significant decrease in the overall number of pulmonary microvessels,[26] which, together with the vasoconstriction and hypertrophic remodeling of the remaining arteries and arterioles, leads to the characteristic decrease in pulmonary vascular conductance observed in this condition. Because the hepatic metabolites of this plant alkaloid[27] have been reported to cause a selective injury to the pulmonary vascular endothelium,[28] it is possible that the mitogenic effects of VEGF accelerated endothelial regrowth and recovery in this model. Alternatively, VEGF, which has also been well characterized as an endothelial cell survival factor,[29,30] may prevent pulmonary microvascular apoptotic cell loss induced by endothelial injury. The demonstration that VEGF gene transfer reduced caspase-3 activation in pulmonary arteriolar endothelium after MCT treatment provides support for this view.

Finally, the well-known angiogenic effects of this growth factor may have contributed to pulmonary microvascular regeneration in the affected lungs and thereby have decreased overall vascular resistance. The efficacy of delayed VEGF gene transfer in established PH is consistent with this possibility; further experiments are necessary, however, to better define which of these mechanisms are involved in the beneficial effects of "angiogenic" gene transfer in the MCT model of PH. Ultimately, the relevance of these findings for patients with arterial PH will need to be determined by appropriately designed clinical studies.

The recent discovery of the genetic defect underlying some cases of familial and sporadic PPH[31–33] may provide new insight into the molecular mechanisms of this disease. A high proportion of individuals exhibited heterozygosity for various mutations in the bone morphogenic protein receptor-2, suggesting that haploinsufficiency or dominant negative protein interactions may lead to a partial inhibition of transforming growth factor-β signaling and thereby contribute to the development of the pathological features of PPH. Among other things, this mutation may reduce the angiogenic effects of transforming growth factor-β[34] and lead to an overall loss of pulmonary microvessels and the vascular pruning that is characteristic of arterial PH.

In summary, cell-based gene transfer to the pulmonary microvasculature resulted in selective transgene overexpression for periods of up to 4 weeks. Also, VEGF gene therapy using this approach was effective in inhibiting the development and progression of PH and improved vascular and RV remodeling in the MCT model. Therefore, these results suggest that the delivery of angiogenic factors combined with a cell-based method of gene transfer may provide a novel therapeutic strategy for pulmonary vascular disorders, for which at present there are few long-term treatment options.

Acknowledgments

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Adenovirus-Mediated Lung Vascular Endothelial Growth Factor Overexpression Protects against Hypoxic Pulmonary Hypertension in Rats

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Chronic hypoxic pulmonary hypertension (PH) is associated with vasoconstriction and structural remodeling of pulmonary vessels including narrowing of the arterial lumen and loss of distal functional arteries. To test whether lung overexpression of the angiogenic factor vascular endothelial growth factor (VEGF) is beneficial in hypoxic PH, recombinant adenovirus encoding the human VEGF 165 gene under the control of a cytomegalovirus promoter (Ad.VEGF) or control vector containing no gene in the expression cassette (Ad.Null) was administered intratracheally to rats. With Ad.VEGF (10^8 plaque-forming units [pfu]), VEGF protein was present in bronchoalveolar lavage fluid as early as 2 d and until 17 d after gene transfer, but was not detected in serum. Only small patchy areas of mononuclear cells without cell damage, edema, or hemorrhage were observed on lung histology with no significant change in lung permeability. In rats pretreated with Ad.VEGF (10^8 pfu) 2 d before a 2-wk exposure to hypoxia (10% O_2), lower values versus Ad.Null-pretreated controls were found for pulmonary artery pressure (25 ± 1 versus 30 ± 2 mm Hg, $P < 0.05$), right ventricular over left ventricular–plus-septum weight (0.37 ± 0.01 versus 0.47 ± 0.02, $P < 0.001$), normalized wall thickness of 50- to 200-μm vessels ($P < 0.001$), and muscularization of distal vessels ($P < 0.001$). Pretreatment with Ad.VEGF (10^8 pfu) increased endothelial nitric oxide synthase activity in lung tissue and partially restored endothelium-dependent vasodilation in isolated lungs from chronically hypoxic rats, as assessed by improvement of ionophore A23187–induced vasodilation and attenuation of endothelin-1 (300 pmol)–induced vasoconstriction, an effect abolished in the presence of nitro-L-arginine methylester. We conclude that adenoviral-mediated VEGF overexpression in the lungs attenuates development of hypoxic PH, in part by protecting endothelium-dependent function.

Hypoxia-induced pulmonary hypertension (PH) is associated with persistent vasoconstriction and structural remodeling of pulmonary vessels responsible for increased medial thickness of muscular arteries, peripheral extension of arterial muscularization, and increased matrix deposition (1). Subsequently, loss of distal functional pulmonary arteries results in increased pulmonary vascular resistance.

The angiogenic factor vascular endothelial growth factor (VEGF), a homodimeric 34- to 42-kD heparin-binding glycoprotein, is a peptide mitogen specific for endothelial cells that fulfills its function by binding to flt-1 and KDR/flk-1, two highly specific tyrosine kinase receptors expressed almost exclusively on endothelial cells (2). Accumulating evidence indicates that stimulation of angiogenesis under hypoxic and ischemic conditions involves upregulation of VEGF and its receptors by hypoxia (3, 4). In the lung, VEGF is primarily and abundantly expressed by epithelial cells (5). Previous studies performed in chronically hypoxic rats have found increases in lung VEGF and VEGF receptors expression (6, 7). Increased VEGF protein and transcripts have also been found in the plexiform lesions of vessels from patients with primary PH (7). However, results obtained in our laboratory as well as in others failed to demonstrate increased lung VEGF expression during development of hypoxic PH (8, 9). Moreover, in the experimental model of monocrotaline-induced PH, which is associated with intense vascular remodeling, a dramatic decrease in lung VEGF messenger RNA expression was observed (8, 10). Specifically, the role of endogenous VEGF in the development of PH and attendant vascular remodeling remains unclarified. In addition to its well-known angiogenic properties, VEGF has been shown to protect against endothelial vascular injury and to improve endothelial function (11–13). Our previous finding of impaired endothelium-dependent relaxation in chronic hypoxic PH (14) invited an investigation of whether lung VEGF overexpression can protect against hypoxic PH and alter the development of pulmonary vascular remodeling.

Gene therapy may be a valuable therapeutic approach in PH. Several studies using intratracheal administration of adenovirus vectors have shown that transgene expression is located mainly in epithelial cells (15, 16). Using this route of administration, previous studies demonstrated that adenoviral-mediated gene transfer of human endothelial nitric oxide synthase (eNOS) in rats was associated with a reduction in acute pulmonary vasoconstriction (17). We therefore reasoned that overexpression of a secreted and diffusible form of VEGF ($VEGF_{165}$) in epithelial cells after adenoviral-mediated gene transfer may affect endothelial cell behavior and protect against pulmonary vascular remodeling during development of hypoxic PH. To investigate this hypothesis, we used a previously described adenovirus vector containing an expression cassette with the cytomegalovirus (CMV) early/intermediate promoter/enhancer driving the human $VEGF_{165}$ complementary DNA (cDNA) (Ad.VEGF) (18).

In the first part of this study, we evaluated the efficiency of gene transfer after a single intratracheal instillation of Ad.VEGF by measuring levels of VEGF protein in

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Abbreviations: bronchoalveolar lavage, BAL; BAL fluid, BALF; endothelial NOS, eNOS; endothelin, ET; nitro-L-arginine methylester, L-NAME; left ventricle, LV; LV plus septum, LV+S; nitric oxide, NO; NO synthase, NOS; plaque-forming units, pfu; pulmonary hypertension, PH; right ventricle, RV; vascular endothelial growth factor, VEGF.

bronchoalveolar fluid and serum after various doses of the adenovirus vector and at various times after the instillation. We also evaluated the effect of Ad.VEGF on pulmonary vessel permeability by measuring the extravascular accumulation of radiolabeled albumin corrected for lung blood weight. In the second part of the study, we assessed pulmonary hemodynamics, right ventricular hypertrophy, and pulmonary vascular remodeling in rats pretreated with intratracheal administration of Ad.VEGF 2 d before the start of a 2-wk exposure to normoxia or hypoxia. Finally, to investigate the mechanisms of the protective effect of Ad.VEGF on the development of hypoxic PH, we measured eNOS activity in lung tissue and examined pulmonary vasoreactivity using isolated lungs from normoxic and chronically hypoxic rats pretreated with Ad.VEGF.

Materials and Methods

Recombinant Adenovirus Vectors

The replication-deficient vector Ad.VEGF is an E1a$^-$, partial E1b$^-$, partial E3$^-$ Ad vector with an expression cassette in the E1 position containing the CMV immediate early promoter/enhancer driving the cDNA for the 165-residue form of human VEGF (18). Ad.Null (similar to Ad.VEGF, but with no gene in the expression cassette) was used as a control vector. All adenovirus vectors were propagated in 293 cells, purified by CsCl gradient centrifugation, dialyzed, and stored at −80°C as previously described (19). The titer of each viral stock was determined by plaque assay in 293 cells.

Animals and Delivery of Adenovirus Vectors to the Lungs

Wistar rats (200 to 250 g body weight) were used for all studies. All animal care and procedures were in accordance with institutional guidelines. Ad.VEGF, or Ad.Null as the control, was diluted before use with sterile saline, pH 7.4, in a final volume of 150 μl. Rats were anesthetized with intraperitoneal ketamine (7 mg/100 g) and xylazine (1 mg/100 g). Intratracheal instillation of 150 μl/rat of diluted Ad.VEGF or Ad.Null was performed using a standard procedure, as previously described (16).

Evaluation of Gene Transfer

Human VEGF protein detection in serum and bronchoalveolar lavage fluid. To evaluate gene transfer efficiency, serum and bronchoalveolar lavage fluid (BALF) levels of human VEGF protein were measured in normoxic rats 5 d after administration with various doses of Ad.VEGF (10^8 to 5×10^9 plaque-forming units [pfu]).

Human VEGF protein was also measured in serum and BALF from normoxic rats 2, 5, 10, and 17 d after administration with Ad.VEGF or Ad.Null (10^8 pfu).

After intraperitoneal administration of pentobarbital (60 mg/kg), blood samples were drawn from the abdominal aorta. Sera obtained after centrifugation of clotted blood samples at 2,000 rpm for 10 min were stored at −20°C. Bronchoalveolar lavage (BAL) was performed immediately after blood sampling. A total of 25 ml of warm phosphate-buffered saline (PBS) was used for each rat. Five aliquots of 5 ml were separately instilled, recovered, and pooled. The supernatants of BALF samples spun at 2,000 rpm, 4°C, for 15 min, were stored at −20°C.

Enzyme-linked immuno-assay (ELISA) detection of human VEGF was carried out on serum and BALF samples using Quantikine (R&D Systems, France). The assay was carried out according to the supplier's instructions, and absorbance at 490 nm was determined on a plate reader.

Histologic evaluation of inflammation after gene transfer. To evaluate the inflammatory response after adenovirus administration, histologic examination was also performed in normoxic rats at various times after treatment with Ad.VEGF or Ad.Null (10^8 pfu) and 5 d after various doses of Ad.VEGF (as explained earlier).

Immediately after BAL, the lungs were removed and fixed by infusion of neutral buffered formaldehyde into the trachea at 25 cm H_2O. After routine processing and paraffin-embedding, multiple sections from each lobe were stained with hematoxylin and eosin. The inflammatory response was analyzed using a previously described empiric semiquantitative scale (16) based on inflammatory cell type and location (alveoli, bronchi, blood vessels) and on the presence of edema and hemorrhage. Epithelial damage in bronchi, bronchioles, or alveoli was scored 0 to 4 (absent to severe, respectively). Extension of inflammation was also scored 0 to 4 as follows: 0, none; 1, small patchy areas involved; 2, < 10% of section area; 3, 10 to 50%; 4, > 50%.

Effect of Ad.VEGF on pulmonary vascular permeability. To evaluate the effect of Ad.VEGF on vascular permeability, rats were administered Ad.VEGF or Ad.Null (10^8 pfu) and then exposed to normoxia ($n = 12$) or hypoxia ($n = 12$). The extravascular accumulation of radiolabeled albumin was measured 5 d after gene transfer. The technique has been previously described (20). In brief, blood was obtained from donor rats and centrifugated. The pellet was incubated at 37°C with [^{51}Cr] for 30 min and then washed twice with saline. Red blood cells were resuspended in plasma. A mixture of a radiolabeled [^{125}I]albumin (1 μCi in 200 μl) and blood containing the [^{51}Cr]-labeled red blood cells (5 to 10 μCi in 300 μl) was injected into the jugular vein of rats anesthetized with ketamine and xylazine. At 1 h later, the chest was opened and a blood sample obtained by cardiac puncture before removal of the lung. The lung and blood samples were weighed and counted in a multichannel scintillation counter. Extravascular accumulation was calculated as whole-lung [^{125}I] counts minus intravascular-lung [^{125}I] counts, where intravascular [^{125}I] counts = (blood [^{125}I] activity) × ([^{51}Cr] counts in lung/blood [^{51}Cr]). The extravascular-lung [^{125}I] count was then divided by intravascular radiolabeled protein concentration (blood [^{125}I] activity) and lung blood weight ([^{51}Cr] count in lung/blood [^{51}Cr] activity) to calculate a protein leak index that should minimize the effects of differences in vascular surface area and relative lung mass.

Effect of Ad.VEGF Pretreatment on Hypoxic PH

To examine the effect of Ad.VEGF administration on hypoxic PH, Ad.VEGF or Ad.Null was administered intratracheally 2 d before the beginning of exposure to normoxia or hypoxia. Hemodynamic measurements and assessment of right ventricular hypertrophy and pulmonary vascular remodeling were performed after 15 d of continuous exposure to normoxia or hypoxia (17 d after gene transfer).

Exposure of rats to chronic hypoxia. Rats were exposed to chronic hypoxia (10% O_2) in a ventilated chamber (500-liter volume; Flufrance, Cachan, France), as previously described (14).

Hemodynamic measurements and assessment of right ventricular hypertrophy. At the end of the 2-wk exposure to normoxia or hypoxia, the rats were anesthetized using an intramuscular injection of ketamine (7 mg/100 g) and xylazine (1 mg/100 g). After exposure of the right jugular vein, a polyvinyl catheter was inserted and manipulated through the right ventricle (RV) into the pulmonary artery. A polyethylene catheter was inserted into the right carotid artery. Pulmonary and systemic arterial pressures were measured under normoxic breathing conditions, immediately after insertion of the catheters, using Gould P 23 ID transducers coupled to pressure modules and a Gould TA 550 multichannel recorder. Only pulmonary artery pressures successfully recorded within 30 min of catheter insertion were taken into account. In some animals, blood was also sampled from the systemic artery catheter for hematocrit measurement. Finally, after an intraperi-

toneal injection of pentobarbital sodium (60 mg/kg), the thorax was opened, the heart was excised and weighed, and the ratio of the weight of the right ventricular free wall to the weight of the septum plus the left ventricular free wall was estimated.

Assessment of pulmonary vascular remodeling. After BAL was performed as previously described, the lungs were fixed in the distended state by infusion of formalin into the trachea. A midsaggital slice of the right lung was processed for paraffin embedding and sections stained with hematoxylin-phloxin-saffron and orcein-picroindigo-carmine. In each rat, a total of 50 to 65 intraacinar vessels accompanying either alveolar ducts or alveoli were analyzed by an observer blinded to the treatment. Each vessel was categorized as muscular, partially muscular, or nonmuscular as previously described (21). The external diameter (the distance between and including the two external elastic laminae intersected by the diameter) and medial thickness (the distance from the luminal surface of the internal elastic lamina to the abluminal surface of the external lamina) were recorded for all muscular and partially muscular arteries. Normalized wall thickness (WT_N) was calculated using the following formula: WT_N (%) = ([2 × medial thickness] ÷ [external diameter]) × 100. The ratio of the number of distal arteries (50 to 200 μm) over the number of alveoli was also assessed on each section.

Pharmacologic Studies in Isolated Lungs from Normoxic and Chronically Hypoxic Rats

Isolated lungs were perfused through a pulmonary arterial cannula using a peristaltic pump at a constant flow of 0.05 ml/g body weight/min with a recirculated physiologic salt solution containing Ficoll (4 g/100 ml, type 70; Sigma Chemical Co., St. Louis). At the start of some experiments, meclofenamate (3.2 μM) was added to the perfusate to achieve complete cyclooxygenase inhibition. Each lung preparation was used for only one of the following procedures.

Effect of exogenous VEGF in isolated lungs from normoxic and chronically hypoxic rats (2 wk). We examined the effect of exogenous VEGF or its vehicle in preconstricted isolated lungs from normoxic (n = 4) and chronically hypoxic (n = 4) rats. After a 30-min equilibration period, the lungs were preconstricted with the endoperoxide analog U-46619, diluted in a 20-ml volume of physiologic salt solution, and infused into the pulmonary arterial line at a constant rate of 50 pmol/min. Pulmonary artery pressure increased gradually in response to U-46619 and did not reach a plateau. Increasing doses (10, 50, 100, and 250 ng) of recombinant human VEGF (Sigma) diluted in distilled water were injected as 50-μl boluses at 3-min intervals into the pulmonary arterial line during U-46619 infusion when the increase in pulmonary arterial pressure reached 6 to 7 mm Hg. These experiments were performed without meclofenamate.

Assessment of vascular reactivity in lungs from rats pretreated with Ad.VEGF or Ad.Null (10^8 pfu) before exposure to normoxia or chronic hypoxia. The effect of the endothelium-dependent vasodilator agent ionophore A23187 and the vasoconstrictor response to endothelin (ET)-1 were assessed in isolated lungs from rats pretreated with Ad.VEGF or Ad.Null (10^8 pfu) 2 d before the beginning of a 2-wk exposure to normoxia or hypoxia. These experiments were performed in the presence of meclofenamate as previously described (14).

The vasodilator effect of ionophore A23187 or its vehicle was tested in lungs preconstricted with U-46619 as described earlier. Ionophore diluted in solutions of increasing concentrations was injected as 50-μl bolus in the perfusate reservoir at 3-min intervals to obtain increasing final concentrations in the recirculating perfusate ($10^{-8.5}$ to 10^{-7} mol/liter).

The vasoconstrictor effect of ET-1 was tested in lungs under baseline conditions. After 30 min of equilibration, ET-1 was injected into the arterial line as a 50-μl bolus containing 300 pmol. The pressor response to ET-1 was measured 20 min after administration. To assess the ability of endothelial cells to produce nitric oxide (NO) in response to ET-1, we also examined the effects of the nitric oxide synthesis (NOS) inhibitor nitro-L-arginine methylester (L-NAME) (5×10^{-4} mol/liter) on ET-1–induced vasoconstriction. The antagonist was added to the perfusate reservoir 10 min before administration of ET-1.

Assessment of eNOS Activity in Lungs from Rats Pretreated with Ad.VEGF or Ad.Null (10^8 pfu)

eNOS activity was assessed in lung tissue from rats that had received intratracheal Ad.VEGF or Ad.Null (10^8 pfu) 2 d before the beginning of a 2-wk exposure to either normoxia or hypoxia. Rats without adenovirus administration (sham) were also studied. On removal, lungs were quickly frozen in liquid nitrogen. Tissue was homogenized on ice using an Ultraturax homogenizer in 4 vol of buffer containing 50 mmol/liter Tris-HCl (pH 7.4), 0.1 mmol/liter ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/liter ethyleneglycol-*bis*-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 0.1% 2-mercaptoethanol, 1 μmol/liter leupeptin, 1 μmol/liter pepstatin A, and 1 mmol/liter phenylmethylsulfonyl fluoride

TABLE 1

Dose-dependent VEGF protein expression and lung histopathology at Day 5 after infection in normoxic rats

	Ad.Null 10^8 pfu (n = 6)	Ad.VEGF 10^8 pfu (n = 7)	Ad.VEGF 5×10^8 pfu (n = 3)	Ad.VEGF 1.5×10^9 pfu (n = 3)	Ad.VEGF 5×10^9 pfu (n = 3)
VEGF in BALF (pg/ml)	0	1,630 (208–3,824)	4,825 (4,701–5,000)	> 5,000	> 5,000
VEGF in serum (pg/ml)	0	0	38 (0–114)	151 (0–241)	670 (83–1,096)
Type of inflammatory cells	Macrophages	Macrophages	Macrophages	Macrophages, lymphocytes	Macrophages, lymphocytes
Inflammation extent (0–4)*	1	1	2/3	2/3	3/4
Edema	0	0	0	1	2
Hemorrhage	0	0	0	1	2
Epithelial damage	0	0	Alveolar	Alveolar and bronchiolar	Alveolar and bronchiolar

Results of VEGF levels are given as mean and range. n indicates number of animals.

*The extent of inflammation is expressed as 1 (small patchy area), 2 (< 10% of section area), 3 (10–50%), 4 (> 50%). Edema, hemorrhage, and epithelial damage were scored 0 to 4 (none to severe).

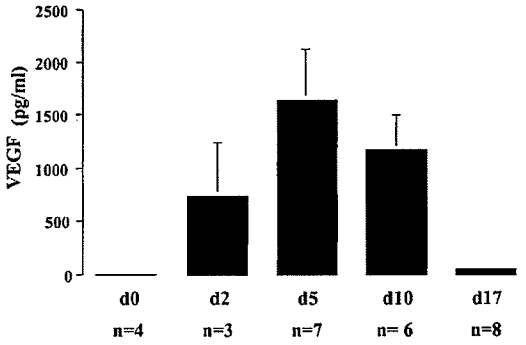

Figure 1. Time-course of human VEGF protein expression in BALF from normoxic rats treated with Ad.VEGF (10^8 pfu). At 0, 2, 5, 10, and 17 d after intratracheal administration of Ad.VEGF (10^8 pfu), BAL was performed using warm PBS with a final volume of 25 ml for each rat. The human VEGF protein level in BALF was determined by ELISA. *n* indicates number of animals studied at each time.

TABLE 2

Body weight, heart weight, and hemodynamic data after 2 wk of normoxia or hypoxia in rats pretreated with adenovirus

	Normoxia		Hypoxia	
	Ad.Null ($n = 7$)	Ad.VEGF ($n = 8$)	Ad.Null ($n = 8$)	Ad.VEGF ($n = 12$)
Final body weight (g)	394 ± 10	370 ± 7	290 ± 6	297 ± 6
RV/BW, × 10^4	5.3 ± 0.2	5.3 ± 0.1	8.7 ± 0.2	6.9 ± 0.2**
LV/BW, × 10^4	18.7 ± 0.6	18.9 ± 0.5	18.5 ± 0.4	18.6 ± 0.3
Pap (mm Hg)	17.1 ± 0.6	16.6 ± 1.0	30.0 ± 2.2	24.8 ± 1.2*
Sap (mm Hg)	107 ± 9	106 ± 7	95 ± 7	96 ± 5
HR (beats/min)	300 ± 13	303 ± 18	305 ± 22	313 ± 12

All values are means ± SEM. BW, body weight; RV, right ventricle weight; LV, left ventricle weight; Pap, pulmonary arterial pressure; Sap, systemic arterial pressure; HR, heart rate. *n* = number of animals studied. Pretreatment with Ad-VEGF significantly attenuated RV/BW and Pap in hypoxic rats but had no effect in normoxic rats.

*$P < 0.05$ and **$P < 0.001$ for comparison with respective controls treated with Ad.Null.

as previously described (22). The homogenate was centrifuged at 100,000 × *g* for 1 h at 5°C. To remove soluble proteins, the pellet was resuspended in homogenization buffer containing 1 mol/liter KCl and allowed to stand on ice for 5 min before centrifugation at 100,000 × *g* for 30 min at 5°C. The supernatant fraction was discarded and the pellet (membrane fraction) resuspended in homogenization buffer containing the detergent CHAPS (20 mmol/liter), 1 mol/liter KCl, and glycerol (10% vol/vol) and allowed to stand on ice for 30 min before centrifugation at 100,000 × *g* for 30 min at 5°C.

Activity of eNOS in the resultant supernatant fraction (membrane fraction) was determined by measuring the Ca^{2+}-dependent conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in the reaction mixture. The enzyme extract (25 μl) was added to 200 μl of the reaction mixture containing 50 mmol/liter Tris-HCl (pH 7.4), 10 μmol/liter tetrahydrobiopterin, 1 mmol/liter dithiothreitol, 10 μg/ml calmodulin, 4 μmol/liter flavin adenine dinucleotide, 4 μmol/liter Flavin Mononucleotide, 2 μmol/liter L-arginine, 1 Kcpm/μl L-[^{3}H]arginine, and 1 mmol/liter nicotinamide adenine dinucleotide phosphate with or without 1 mmol/liter $CaCl_2$. After 40 min of incubation at 37°C, the reaction was stopped with 2 ml of a solution containing 20 mmol/liter Na acetate pH 5.5, 1 mmol/liter L-citrulline, 2 mmol/liter EDTA, and 0.2 mmol/liter EGTA. The mixture was applied to a 1-ml Dowex AG 50WX8 column and L-[^{3}H]citrulline was eluted with 2 ml of distilled water. The radioactivity in the eluate was measured by liquid scintillation spectroscopy. The concentration of protein in the enzyme extract was determined according to Lowry.

Statistical Analysis

All results are expressed as means ± standard error of the mean (SEM). A two-way analysis of variance (ANOVA) was performed to compare the effect of Ad.VEGF versus Ad.Null pretreatment in normoxic and hypoxic rats, followed by Fisher's test or nonparametric Mann–Whitney test to compare Ad.VEGF and Ad.Null pretreatment for each condition of oxygenation when interaction was significant. To compare the degrees of pulmonary vessel muscularization in the two groups of rats, vessels were ordinally classified as nonmuscular, partially muscular, and muscular. Comparison of muscularization between two groups was performed separately at the alveolar duct and wall levels using a nonparametric Mann–Whitney test. To compare the effect of ionophore A23187 versus its vehicle in isolated rat lungs, two-way ANOVA with repeated measurements was performed; followed, because interaction was significant, by nonparametric Mann–Whitney test to compare drug versus vehicle at each dose.

Results

Evaluation of *In Vivo* Gene Transfer in Normoxic Rats

Dose-dependent protein expression and inflammatory response 5 d after adenovirus administration. As shown in Table 1, as little as 10^8 pfu of Ad.VEGF produced detectable amounts of human VEGF protein in BALF. With increasing amounts of Ad.VEGF, human VEGF protein increased in a dose-dependent manner in BALF and became detectable in serum. The intensity of the inflammatory response also varied in a dose-dependent manner. With 10^8 pfu of either Ad.VEGF or Ad.Null, inflamma-

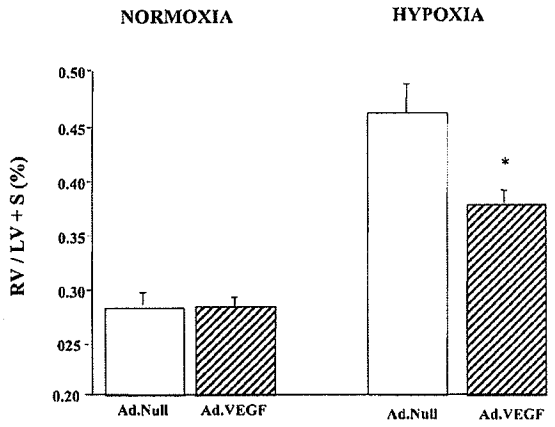

Figure 2. Ratio of RV to LV plus septum weight (RV/LV+S) in rats exposed to normoxia (*left*) or chronic hypoxia (2 wk; *right*) and pretreated with either Ad.VEGF or Ad.Null. Pretreatment with Ad-VEGF significantly attenuated RV/LV+S in hypoxic rats but had no effect on normoxic rats. *$P < 0.05$ for comparison with corresponding values obtained in rats pretreated with Ad.Null.

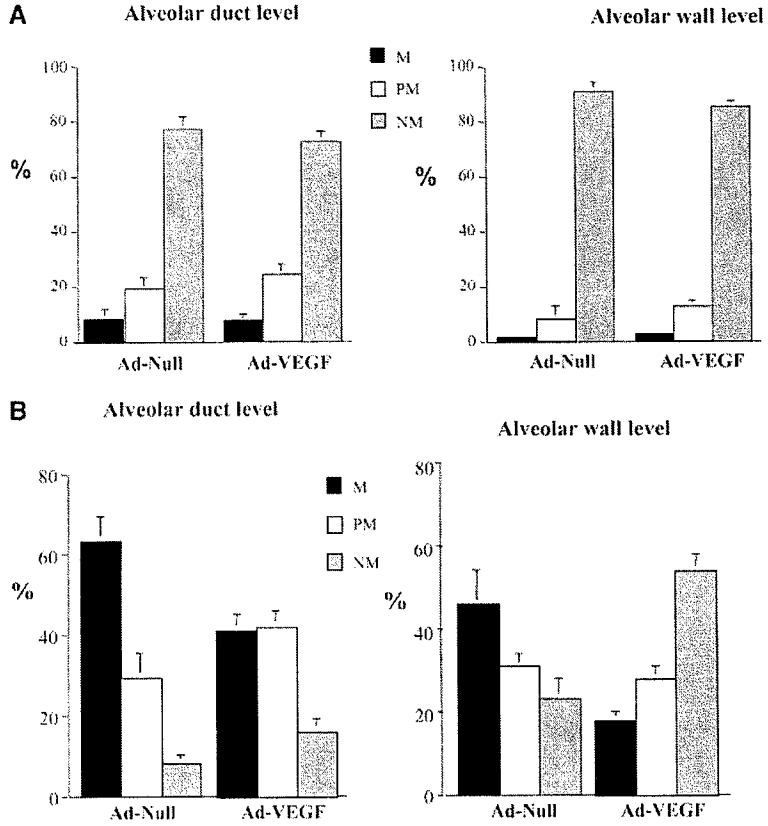

Figure 3. Percentage of non- (NM), partially (PM), or fully muscularized (M) vessels according to the accompanying airway. A total of 50 to 65 intraacinar vessels was analyzed in each lung from rats exposed to normoxia (*A*) or 2-wk hypoxia (*B*) and pretreated with either Ad.VEGF or Ad.Null. After exposure to chronic hypoxia, muscularization at alveolar duct or alveolar wall level was significantly lower after Ad.VEGF than Ad.Null pretreatment ($P < 0.001$, nonparametric Mann–Whitney test on ordinally classified vessels). Pretreatment with Ad-VEGF did not affect muscularization of pulmonary vessels in normoxic rats.

tion was characterized by patchy infiltrates of mononuclear cells, most of which were macrophages. No cell damage, edema, or hemorrhage was observed. Higher doses of Ad.VEGF produced a more diffuse inflammatory response, accompanied in some cases by mild cell damage in the alveolar and bronchiolar epithelium, edema, and hemorrhage. These changes likely reflected the tissue response to the viral vectors and were consistent with previous findings (15, 16). On the basis of this dose-response experiment, 10^8 pfu was the dose selected for further experiments because it caused minimal inflammatory responses but significant human VEGF protein production in lungs without detectable levels in serum. After treatment with Ad.Null (10^8 pfu), no human VEGF was detected in BALF or serum.

Time-course of protein expression after adenovirus administration (10^8 pfu). Expression of human VEGF protein in BALF samples was detectable on Day 2 and peaked on Day 5 after treatment with Ad.VEGF (10^8 pfu) (Figure 1). On Day 17, human VEGF protein was still detectable in BALF from five of eight rats.

Effect of Ad.VEGF (10^8 pfu) treatment on pulmonary vascular permeability. There was a trend for an increase in

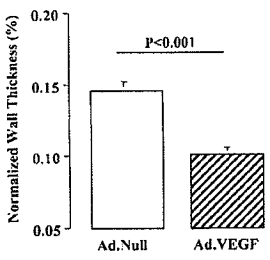

Figure 4. Normalized wall thickness measured in muscular or partially muscular arteries (external diameter of 50 to 200 μm) in lungs from rats exposed to chronic hypoxia (2 wk) and pretreated with either Ad.VEGF ($n = 11$) or Ad. Null ($n = 8$). A total of 50 to 65 intraacinar vessels was analyzed in each rat.

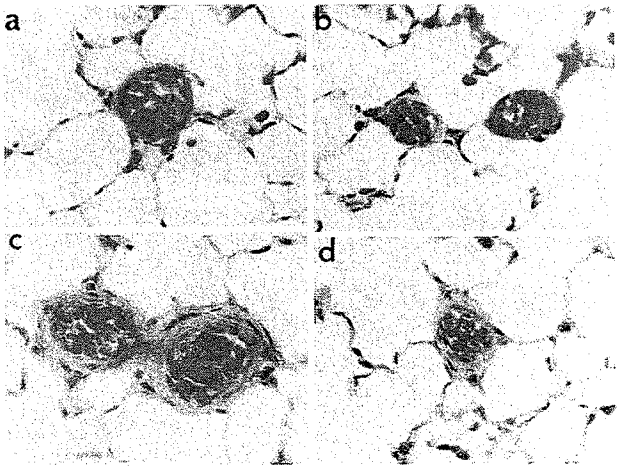

Figure 5. Pulmonary vascular remodeling illustrated by representative photomicrographs of pulmonary vessels from rats exposed to chronic hypoxia (2 wk) and pretreated with either Ad.VEGF (*a* and *b*) or Ad.Null (*c* and *d*). Sections of 5 μm thickness were cut for light microscopy and stained with hematoxylin-phloxin-saffron. Original magnification: ×40: 15 μm represents 50 mm.

TABLE 3

Basal pulmonary perfusion pressure and pressure changes induced by U-46619 and ET-1 in isolated lungs from normoxic and from chronically hypoxic rats pretreated with adenovirus

	Normoxia		Hypoxia	
	Ad.Null	Ad.VEGF	Ad.Null	Ad.VEGF
Basal pulmonary perfusion pressure (mm Hg)	8.7 ± 0.6 (n = 15)	8.2 ± 0.4 (n = 15)	16.1 ± 0.5 (n = 21)	14.6 ± 0.5* (n = 20)
Pressure changes (mm Hg)				
U-46619, 50 pmol/min				
1 min	2.7 ± 0.7 (n = 5)	1.2 ± 0.5* (n = 5)	7.0 ± 1.1 (n = 5)	1.1 ± 0.3** (n = 5)
10 min	15.7 ± 0.8 (n = 5)	8.1 ± 1.0* (n = 5)	> 20 (n = 5)	9.8 ± 0.8** (n = 5)
ET-1 (300 pmol)	3.2 ± 0.5 (n = 5)	3.0 ± 0.3 (n = 5)	13.8 ± 1.8 (n = 7)	6.4 ± 0.6** (n = 5)

All values are means ± SEM. n = number of lungs studied. * $P < 0.05$ and ** $P < 0.001$ for comparison with respective controls treated with Ad.Null. Pretreatment with Ad.VEGF attenuated basal pulmonary perfusion pressure and response to ET-1 in hypoxic rats but had no effect in normoxic rats. The pressor response to U46619 was decreased by pretreatment with Ad.VEGF in both normoxic and hypoxic rats.

pulmonary vascular permeability in hypoxic as compared with normoxic rats, but differences did not reach statistical significance. Transvascular protein escape measured at maximum of protein expression was not altered by Ad.VEGF pretreatment. Indeed, in rats exposed to normoxia, protein leak index was not significantly increased 5 d after gene transfer in Ad.VEGF- as compared with Ad.Null-treated rats (1.62 ± 0.33 versus 1.20 ± 0.15, respectively; not significant [NS]). When rats were exposed to hypoxia immediately after gene transfer, protein leak index also

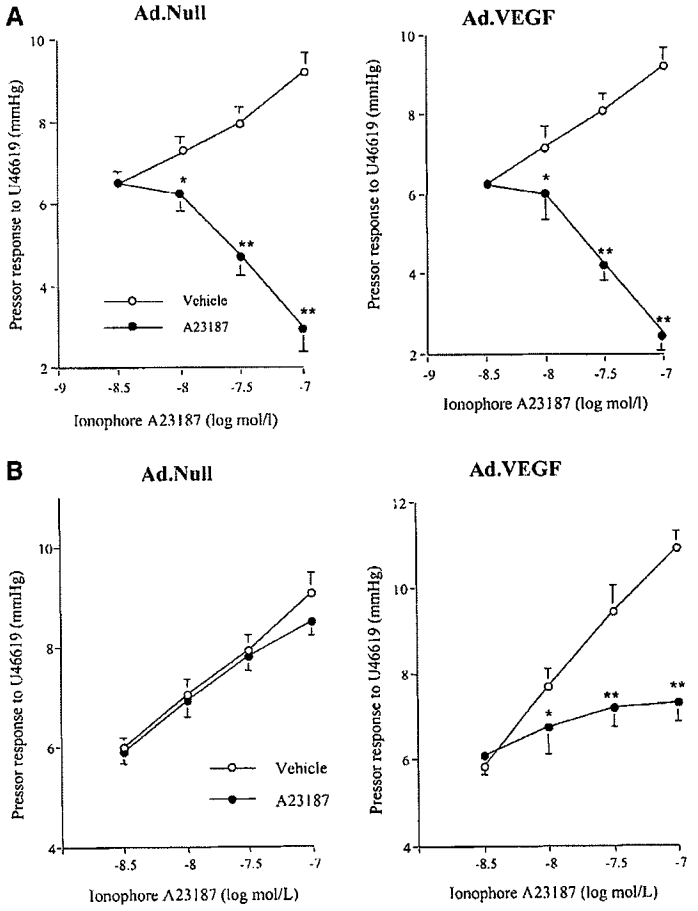

Figure 6. Vasodilatory response to ionophore A23187 during a continuous infusion of U-46619 as indicated by pressure on y axis versus concentration of ionophore on x axis, in lungs from rats exposed to normoxia (A) or chronic hypoxia (B) and pretreated with either Ad.Null or Ad.VEGF. Ionophore A23187 (*filled circles*) or vehicle (diluted ethanol; *open circles*) was administered into the perfusate reservoir as 50 μl of increasing doses (final concentration: $10^{-8.5}$ to 10^{-7} mol/liter) separated by time intervals of 3 min. * $P <$ 0.05, ** $P < 0.01$ for comparison with corresponding time control values measured with vehicle. n = 5 in each experiment.

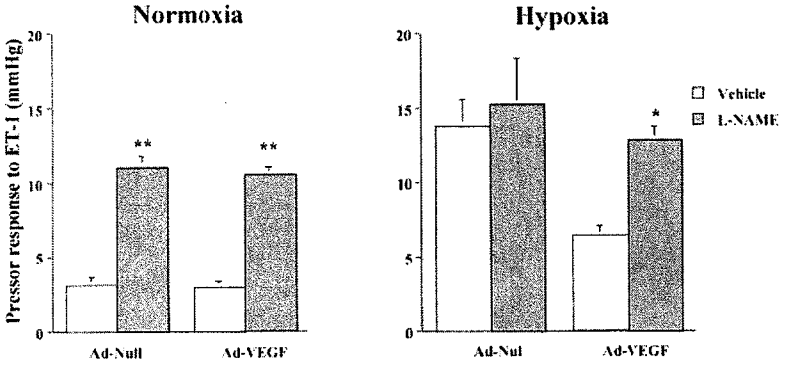

Figure 7. Increase in baseline perfusion pressure in response to ET-1 (300 pmol) in isolated lungs from rats exposed to normoxia (*left*) and to chronic hypoxia (*right*) and pretreated with either Ad.Null or Ad.VEGF. Vasopressor effect of ET was measured in the presence of L-NAME (5×10^{-4} mol/liter), an inhibitor of NOS, or its vehicle (H_2O), which were added to the perfusate reservoir 20 min before the injection of ET-1 ($n = 5$ in each experiment).

did not differ between Ad.VEGF- and Ad Null-pretreated rats (2.09 ± 0.18 and 1.64 ± 0.43, respectively; NS).

Effects of Ad.VEGF Pretreatment on Chronic Hypoxic PH

Administration of either Ad.VEGF or Ad.Null (10^8 pfu) was well tolerated in animals studied during the 17 d. No deaths or symptoms of respiratory failure were observed in rats exposed to normoxia or chronic hypoxia.

Hemodynamic measurements and assessment of right ventricular hypertrophy. In rats exposed to normoxia for 17 d after adenovirus administration, pulmonary artery pressure (Table 2) and RV weight over either body weight or left ventricle (LV) plus septum (LV+S) weight (Figure 2) did not differ between Ad.VEGF- and Ad.Null-treated animals and were similar to values previously reported in normoxic rats.

Exposure to hypoxia for 15 d after adenovirus pretreatment was associated with an increase in pulmonary artery pressure and development of right ventricular hypertrophy in Ad.VEGF and Ad.Null rats. However, in chronically hypoxic rats, pulmonary artery pressure was significantly lower after Ad.VEGF than after Ad.Null pretreatment ($P <$ 0.05), whereas systemic arterial pressure and heart rate were similar (Table 2). Right ventricular hypertrophy as assessed by RV weight over either body weight or LV+S weight (Figure 2) was also less marked in chronically hypoxic rats pretreated with Ad.VEGF than in those given Ad.Null ($P < 0.001$), whereas LV weight was similar in both groups. After exposure to hypoxia, hematocrit was also similar in Ad.VEGF- and Ad.Null-pretreated rats (46 ± 0.1% and 45 ± 0.1%, respectively). Final body weight did not differ between these two groups.

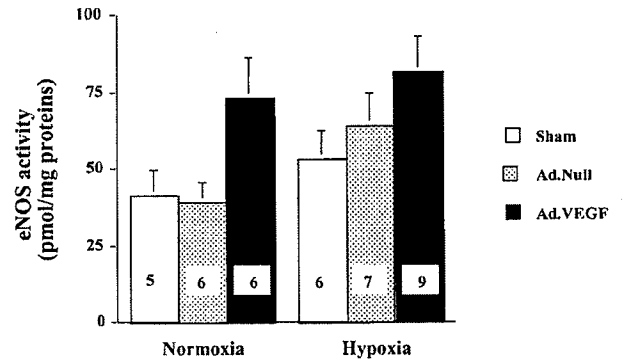

Figure 8. Values of eNOS activity in lung tissue from rats exposed to normoxia or chronic hypoxia (2 wk) without (Sham; *open bars*) or with pretreatment with either Ad.VEGF (*stippled bars*) or Ad.Null (*filled bars*). Numbers in columns indicate numbers of animals studied. Values from Ad.Null and Ad.VEGF differed significantly ($P < 0.01$, Fisher's test) but no differences between Ad.Null and sham or between normoxia and hypoxia, as well as no interaction between pretreatments and exposure, were observed (two-way ANOVA).

Measurement of human VEGF in BALF. Human VEGF protein was still detectable in the BALF from 7 out of 13 of these chronically hypoxic rats studied 17 d after administration of Ad.VEGF. Values ranged from 3 to 1,942 pg/ml.

Structural remodeling of distal pulmonary vessels. In each rat, a total of 35 to 65 intraacinar vessels were examined to determine the percentage of nonmuscularized and partially and totally muscularized vessels according to the accompanying airway (alveolar duct or alveolar wall). In rats exposed to normoxia for 17 d after adenovirus administration, muscularization of distal vessels did not differ between Ad.VEGF- and Ad.Null-treated animals and were similar to values previously reported in normoxic rats (Figure 3A). Pretreatment with Ad.VEGF partially prevented muscularization of distal pulmonary arteries in response to hypoxia at both the alveolar duct and the alveolar wall level (Figure 3B; $P < 0.001$). Moreover, as compared with control animals (Ad.Null) similarly exposed to hypoxia, the normalized wall thickness of muscularized and partially muscularized arteries was markedly reduced in Ad.VEGF-pretreated rats (Figure 4; $P < 0.001$; and Figure 5).

The number of distal vessels (50 to 200 μm) was counted on 10 to 20 sections per rat and normalized for the number of alveoli. After 15 d of hypoxia, no significant difference was observed between the Ad.VEGF-treated group (12.2 ± 0.9 arteries per 100 alveoli) and the Ad.Null group (11.0 ± 0.3), suggesting the absence of new vessel development.

Pharmacologic Studies in Isolated Lungs

Response to exogenous VEGF in isolated lungs from normoxic and chronically hypoxic rats. As compared with vehicle alone, in isolated lungs from either normoxic or chronically hypoxic rats, increasing doses of recombinant human VEGF had no effect on the increase in perfusion pressure induced by continuous infusion of U-46619 (data not shown).

Assessment of pulmonary vascular reactivity in lungs from rats pretreated with Ad.VEGF or Ad.Null (10^8 pfu). Mean baseline pulmonary artery pressure was similar in lungs from rats exposed to 17 d of normoxia after Ad.VEGF or Ad.Null administration.

Exposure to 2 wk of hypoxia (10% O_2) after Ad.VEGF or Ad.Null administration was associated with an increase in mean baseline perfusion pressure as compared with lungs from normoxic rats. However, in lungs from hypoxic rats, perfusion pressure was significantly lower after Ad.VEGF than after Ad.Null pretreatment (Table 3; $P < 0.05$). Moreover, in lungs from normoxic or hypoxic rats, the pressor response after 1 or 10 min of U-46619 infusion (50 pmol/min) was less marked after Ad.VEGF than after Ad.Null pretreatment ($P < 0.001$). In subsequent experiments performed to assess the vasodilator effect of ionophore A23187, the rate of U-46619 infusion in lungs from Ad.Null-pretreated rats was decreased to 25 pmol/min to obtain approximately the same pressure increase in both groups of lungs before ionophore administration.

In lungs from normoxic rats, ionophore A23187 elicited a dose-dependent decrease in perfusion pressure that was similar after Ad.VEGF or Ad.Null pretreatment (Figure 6A). In contrast, ionophore A23187 did not modify the perfusion pressure increase in response to U-46619 in lungs from hypoxic rats pretreated with Ad.Null, whereas it stabilized perfusion pressure despite maintenance of U-46619 infusion (Figure 6B) in lungs from Ad.VEGF-pretreated rats similarly exposed to hypoxia.

Under conditions of baseline tone, ET-1 (300 pmol) induced an increase in perfusion pressure (Figure 7). This increase was similar in lungs from normoxic rats pretreated with Ad.Null or Ad.VEGF. After exposure to hypoxia, the increase in perfusion pressure elicited by ET-1 was larger than in lungs from normoxic rats and also more marked in lungs from Ad.Null than in lungs from Ad.VEGF rats ($P < 0.05$). Pretreatment with the NO synthesis inhibitor L-NAME (5×10^{-4} mol/liter) potentiated the pressor response to ET-1 in lungs from both groups of normoxic rats as well as in lungs from hypoxic rats pretreated with Ad.VEGF, but had no effect in lungs from rats pretreated with Ad.Null and similarly exposed to hypoxia. After L-NAME pretreatment, the pressor response to ET-1 did not differ between lungs of the two groups of hypoxic rats.

Effect of Ad.VEGF Treatment on eNOS Activity

Results of the Ca^{2+}-dependent NOS activity assessed in the membrane fraction of lung tissue after 2 wk of exposure to normoxia or hypoxia are shown in Figure 8. There was no significant effect of hypoxia as compared with normoxia on eNOS activity, which also did not differ between Ad.Null-pretreated rats and those without adenovirus administration (sham) (two-way ANOVA). However, eNOS activity was increased in Ad.VEGF- as compared with Ad.Null-pretreated ($P < 0.05$) or sham ($P < 0.01$) rats.

Discussion

Our findings show that adenoviral-mediated lung VEGF overexpression, which had no effect on the pulmonary circulation during normoxia, protected rats exposed to chronic hypoxia against development of PH. Values were lower for pulmonary arterial pressure, right ventricular hypertrophy, and distal vessel muscularization in hypoxic rats pretreated with Ad.VEGF as compared with rats pretreated with the control vector Ad.Null. Our results also suggest that this protective effect of VEGF on the pulmonary circulation during exposure to chronic hypoxia may be at least partially related to an improvement in endothelial function. Indeed, Ad.VEGF pretreatment was associated with increased lung eNOS activity, partial restoration of the vasodilator response to the endothelium-dependent agent ionophore A23187, and marked blunting of ET-1–induced vasoconstriction.

VEGF is now considered a potent angiogenic mediator that can be overexpressed in target tissues via either a replication-deficient adenovirus vector or naked DNA (23–25). In various models of myocardial or peripheral ischemia, as well as in humans with peripheral arteriopathy, VEGF has been shown to promote revascularization of ischemic tissues by stimulating collateral vessel development (26, 27). Although the mechanisms leading to collateral vessel growth in response to VEGF are not well understood (28), it is now widely accepted that hypoxia is a major stimulus for angiogenic factor expression and activity. In addition to its mitogenic effects on endothelial cells, VEGF has also been shown to improve endothelial function and to diminish systemic artery remodeling (11, 13, 29). However, the effects of VEGF on pulmonary vessels have not been examined, particularly during exposure to hypoxia, which leads to pulmonary vascular remodeling and subsequent development of PH. Increased lung VEGF expression may be desirable during hypoxia-induced PH, not only to promote the development of new pulmonary vessels but also to improve endothelial function. Moreover, the pulmonary arteries affected by remodeling during PH are in close proximity to epithelial cells from distal airways. Transfer of the human $VEGF_{165}$ gene to these cells by intratracheal administration of an adenoviral vector (17, 19) may therefore provide a means of exposing pulmonary vessels to high levels of a diffusible and secreted form of VEGF.

In the present study, adenoviral transfer allowed efficient local overexpression of human $VEGF_{165}$ in rat lungs. After intratracheal administration of Ad.VEGF (10^8 pfu), the $VEGF_{165}$ protein was detected in BALF for about 17 d, with a concentration peak on Day 5. No VEGF was detectable in the serum with this low dose of adenovirus, suggesting that the risk of VEGF diffusion or expression in other organs was minimal. As shown by our histologic study, administration of 10^8 pfu of Ad.VEGF or Ad.Null caused only mild inflammation in lungs from both normoxic and hypoxic rats. Only small patchy areas of macrophage infiltrates were observed, with no epithelial cell damage, edema, or hemorrhage. Moreover, measurement of transvascular protein escape at time of peak expression did not provide evidence for an increased vascular permeability in lungs from rats pretreated with this low dose of Ad.VEGF and subsequently exposed to normoxia or hypoxia.

The main finding from our study is that VEGF overexpression in lung tissue did not affect the pulmonary circulation in normoxic conditions but attenuated the devel-

opment of PH and right ventricular hypertrophy in rats exposed to chronic hypoxia. Pulmonary artery pressure and RV weight were lower in rats pretreated with Ad.VEGF than in hypoxic control rats pretreated with Ad.Null, whereas systemic arterial pressure and LV weight were similar. Attenuation of PH was not related to an effect of VEGF overexpression on hypoxia-induced polycythemia, because hematocrit did not differ between the two groups of adenovirus-infected animals. The development of hypoxic PH is associated with smooth-muscle cell hypertrophy and hyperplasia in normally muscularized arteries and with the appearance of new smooth-muscle cells in nonmuscularized segments of the intraacinar circulation. In addition to decreases in PH and right ventricular hypertrophy, reductions in the percentage of muscularized arteries at the alveolar duct and wall level and in muscularized artery wall thickness were seen in hypoxic rats pretreated with Ad.VEGF. In the aim to explain this protective effect of VEGF overexpression on development of PH, we questioned whether these effects could be due to a direct vasodilator effect of VEGF on the pulmonary circulation. However, in contrast to effects shown in coronary arteries (13), we did not observe any direct vasodilator effect of VEGF in the rat pulmonary circulation. Bolus administration of the recombinant protein did not reverse the vasoconstrictor effect of U-46619 in isolated lungs from either normoxic or chronically hypoxic rats. We also questioned whether lung VEGF overexpression could be associated with an increase in the number of pulmonary arteries. Indeed, newly formed collateral arteries have previously been demonstrated in response to VEGF after occlusion of large systemic arteries in the limb circulation or in the heart (2, 24, 25, 27). In case of development of newly formed pulmonary arteries, one could speculate that VEGF overexpression, even transient, provides long-term protection against PH. In the present study, lung VEGF overexpression was not associated with a significant increase in the number of distal vessels, as assessed by counting the number of distal vessels over the number of alveoli. These results, therefore, differ from those obtained in experimental animal models of local ischemia after treatment with VEGF. It is now established that arteriogenesis depends on a pre-existing network of arterioles that is present in most tissues, especially in the vascular periphery of the limb circulation and the heart (28). Because such an arteriolar network is not present in the lung, arteriogenesis may not develop in response to angiogenic factors in the pulmonary vascular tree. Together, these observations demonstrate that the beneficial effect of VEGF overexpression on PH development was due to diminished vascular remodeling, with no evidence for an increase in the number of pulmonary arteries.

It is now well known that focal vascular injury and impaired endothelial function are important features of PH that lead to enhanced platelet endothelial cell interactions and contribute to thrombosis and smooth-muscle proliferation. Recently, numerous studies have suggested that in addition to its mitogenic effect on endothelial cells, VEGF may act as an endothelial cell "survivor factor" (13, 30). In systemic arteries, increasing VEGF bioavailability at sites of endothelial injury has been shown to accelerate endothelial repair and to limit neointima formation (29, 30). Moreover, VEGF overexpression within the vascular wall has been shown to restore endothelial-dependent relaxation and to protect against vasoconstriction (11).

In the present study, VEGF overexpression improved endothelium-dependent vasodilation and blunted vasoreactivity to the constrictor agents U-46619 and ET-1 in lungs from chronically hypoxic rats. We have previously shown that endothelial function is impaired in the pulmonary circulation from rats exposed to chronic hypoxia (14). The vasodilator response to acetylcholine or ionophore A23187, two endothelium-dependent agents, is abolished in isolated lungs from rats previously exposed for 3 wk to severe hypoxia. In the present study, we found that VEGF overexpression did not affect endothelium-dependent vasodilation to ionophore A23187 in lungs from normoxic rats but partially restored it after exposure to chronic hypoxia. Moreover, the vasoconstrictor response to ET-1, which was not affected by VEGF overexpression in lungs from normoxic rats, was significantly increased in lungs from hypoxic rats. However, in rats similarly exposed to hypoxia, the pressor response to ET-1 was less marked in Ad.VEGF- than in Ad.Null-pretreated rats. It is noteworthy that pressor response to ET-1 in Ad.VEGF-pretreated hypoxic rats was potentiated after L-NAME pretreatment, reaching the same magnitude as in lungs from Ad.Null rats exposed to a similar degree and duration of hypoxia. In conjunction with the later results, we also found that lung eNOS activity was enhanced in lungs from animals treated with Ad.VEGF as compared with those treated with Ad.Null. Altogether, these findings demonstrate that VEGF overexpression in the lungs led to protection of endothelial function and enhanced release of endothelial NO formation which may have contributed to attenuation of hypoxia-induced pulmonary vascular remodeling.

The reasons for the impaired NO-mediated vasodilation during chronic hypoxia-induced PH are not yet completely understood inasmuch as eNOS protein expression and activity have been shown to be unchanged or increased in lung tissue from chronically hypoxic rats (22, 31). These paradoxical findings clearly appeared in the present study showing abolition of ionophore A23187–induced vasodilation despite no decrease in eNOS activity. Thus, in chronic hypoxic PH, eNOS, although present in increased amounts, may produce insufficient NO to oppose hypoxia-induced constriction and associated vascular remodeling. Our previous results showing protection against hypoxia-induced PH by continuous inhalation of NO are consistent with this hypothesis (21). Despite our observation that acute supplementation of L-arginine improves endothelium-dependent NO formation in lungs from chronically hypoxic rats, long-term improvement of endothelial NO formation could not be achieved by chronic L-arginine treatment (32). Numerous studies have shown that VEGF increases production of NO in systemic arteries. However, the mechanism of this effect remains presently unclear. VEGF may interfere with eNOS at several levels, including interaction with other membrane proteins such as caveolin, protein trafficking, or substrate availability in the vicinity of the enzyme (33, 34). In a recent study, VEGF has also been shown to increase eNOS expression

via activation of the KDR receptor tyrosine-kinase and a downstream protein kinase C signaling pathway (35). The signaling pathway between VEGF and eNOS as well as other regulators of eNOS is probably not specific for the pulmonary circulation.

Our finding that VEGF overexpression in the lungs obtained by means of adenovirus-mediated gene transfer improves endothelial function and attenuates development of PH and vascular remodeling in a rat model of hypoxic PH may have clinical implications regarding the treatment of PH. However, further studies are needed to determine whether VEGF overexpression can also reverse established PH and whether it also plays a beneficial role in other models of PH. The principal limitation of adenoviral vector–mediated transfer may be the limited duration of VEGF expression in lung tissue. However, rather than a limitation, this feature may be an advantage in some situations where expression during only a few weeks may provide both adequate therapeutic efficacy and limited side effects. Development of second-generation adenoviral vectors will provide new tools to prolong the duration of transgene expression.

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